The antimicrobial mechanism of action of 3,4-methylenedioxy-β-nitropropene

A thesis submitted in fulfillment of the requirements for the degree of Doctor of Philosophy

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Declaration

I certify that except where due acknowledgement has been made, the work is that of the author alone; the work has not been submitted previously, in whole or in part, to qualify for any other academic award; the content of the thesis is the result of work which has been carried out since the official commencement date of the approved research program; and, any editorial work, paid or unpaid, carried out by a third party is acknowledged.

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LIST OF ABBREVIATIONS

AAF	Alanine-alanine-phenylalanine
AATS	Aminoacyl-tRNA synthesis
ABC	ATP-binding cassette
ADP	Adenosine diphosphate
AGRF	Australian Genome Research facility
AHL	*N*-acyl homoserine lactone
AMC	Aminomethyl coumarin
AOAC	Association of Official Analytical Chemists
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
BB	Brucella broth
BDM-I	3,4-methylenedioxy-β-nitropropene
cDNA	Complementary deoxyribonucleic acid
CFU	Colony forming units
CPS	Capsullar polysaccharide
C_t	Cycle threshold
°C	Degrees Celsius
DEPC	Diethylene Pyrocarbonate
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DSP	Dual Specific Phosphatase
DTT	Dithiothreitol
EB	Elementary body
EDTA	Ethylenediaminetetraacetic acid
FICI	Fractional Inhibitory Concentration Index
GCOS	GeneChip® Operating Software
h	Hour/s
H_2O_2	Hydrogen peroxide
HBA	Horse blood agar
HCl	Hydrochloric acid
HEPES	N-(2-hydroxyethyl)-piperazine-N'-2-ethane sulfonic acid
IC_{50}	Inhibitory concentration, 50%
JCVI CMR	The J. Craig Venter Institute Comprehensive Microbial Resource
kg	Kilogram/s
L	Litre/s
LB	Luria broth
LBA	Luria broth agar

LD_{50}	Lethal dose, 50%
LMW PTP	Low molecular weight protein tyrosine phosphatase
LOS	Lipo-oligosaccharide
LPS	Lipopolysaccharide
LU	Luminescence Units
McF0.5	MacFarland opacity standard 0.5
MDR	Multi-drug resistant
MEM	Minimum essential media
MES	2-morpholinoethanesulfonic acid, monohydrate
MHB	Mueller Hinton broth
MIC	Minimum inhibitory concentration
min	Minute/s
mg	Milligram/s
µg	Microgram/s
mL	Millilitre/s
µL	Microlitre/s
M	Molar concentration
mM	MilliMolar
MM	Maintenance medium
MMC	Minimum microbicidal concentration
MOA	Mechanism of action
mRNA	Mitochondrial ribonucleic acid
MW	Molecular weight
NA	Nutrient agar
NCCLS	National Committee on Clinical Laboratory Standards
ng	Nanogram/s
NOAEL	No observed adverse effect
NPN	1-N-phenylnapthylamine
OD	Optical density
OM	Outer membrane
ONP	*o*-nitrophenyl
ONPG	*o*-nitrophenyl–β-D-galactopyranoside
ORF	Open reading frame
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PG	Prodigiosin
PGB	Peptone glycerol broth
PK	Protein kinase
PNPG	ρ-nitrophenylglycerol
PP	Protein phosphatase
PSTP	Protein serine threonine phosphatase

PTK	Protein tyrosine kinase
PTP	Protein tyrosine phosphatase
qPCR	Quantitative PCR
QS	Quorum sensing
R110	Rhodamine 110 peptide
RB	Reticulate body
RMIT	RMIT University (Melbourne, Australia)
RNA	Ribonucleic acid
RNAP	RNA polymerase
RNase	Ribonuclease
rpm	Revolution/s per minute
rRNA	Ribosomal ribonucleic acid
RT	Reverse transcription
σ	Sigma
SAPE	Streptavidin-phycoerythrin
SA-PTP	Small acidic PTP
SCV	Small colony variant
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate Polyacrylamide gel electrophoresis
SLM	Sabouraud liquid medium
spp.	Species
TARP	Tyrosine actin recruiting phosphoprotein
TE	Tris EDTA buffer
TEM	Transmission electron microscopy
T_m	Melting temperature
Tris	2-Amino-2-(hydroxymethyl)-1,3-propanediol
tRNA	Transfer ribonucleic acid
TTSS	Type three (III) secretion system
TGT	Target intensity value
U	Unit/s
UV	Ultraviolet
w/v	Weight per volume
YNB	Yeast nitrogen broth

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ABSTRACT

This research investigated the mechanism of action in bacteria of 3,4-methylenedioxy-β–nitropropene (BDM-I), a very broad spectrum antimicrobial lead compound in development as an anti-infective drug. The thesis proposes that BDM-I inhibits bacterial protein tyrosine phosphatases, a novel mechanism of action for an antimicrobial agent and a new target in microorganisms.

This very open investigation was directed by considerable biological information on the effects of BDM-I in microorganisms and animals which provided insights into possible and improbable cellular targets. The biological effects of BDM-I were investigated using biochemical and cell-based assays, transmission electron microscopy and whole genome DNA microarray analysis. The specific experiments and order of execution were largely dependent on information gained as the project progressed.

BDM-I was shown not to target the metabolic pathways of the major classes of antibacterial drugs, which supports a novel mechanism of action. Investigation of several species-specific effects suggested that cell signalling pathways were a possible target. Based on the structure of BDM-I and review of the scientific literature on cell signalling in bacteria, the hypothesis that BDM-I acted by inhibition of protein tyrosine phosphatases (PTP) was supported by demonstrating inhibition of human and bacterial PTP's in an enzyme assay. This mechanism was consistent with other demonstrated effects: inhibition of the intracellular pathogen, *Chlamydia trachomatis*; inhibition of swarming in *Proteus* spp. and inhibition of pigment production in *Serratia marcescens;* and with kill kinetics in bacteria and yeast.

A pilot global genome analysis of BDM-I treated *Bacillus subtilis* did not detect differential expression of PTP genes but has provided many avenues for further investigation.

This research further supports the development of BDM-I as a broad spectrum anti-infective drug.

CHAPTER 1 GENERAL INTRODUCTION

1.1 AIM OF THIS RESEARCH

The aim of the project was to identify the mechanism(s) of action of a novel small molecule synthetic antimicrobial compound, BDM-I (3,4-methylenedioxy-β-nitropropene), in bacteria. Given its broad activity against bacteria, fungi and protozoa, a very general major mechanism against a target highly conserved across microbial types was hypothesised. This was thought more likely than multiple mechanisms of action against the different susceptible microbial types. It was further hypothesised that BDM-I would be unlikely to act on the metabolic targets of the common antibacterial and antifungal agents which are more specific to microbial types. The hope was to discover a novel bacterial target which was also common to eukaryotic unicellular organisms. A more modest hope was to discover additional mechanisms specific to certain bacterial types which would explain idiosyncratic responses by certain species to BDM-I.

The biological profile of BDM-I described below (Section 1.2), supported the belief that BDM-I might target a novel pathway. The very broad spectrum activity against phenoytypes resistant to common antimicrobials and the lack of development of phenotypes resistant to BDM-I *in vitro* all suggest a new but widely used and important metabolic target common to unicellular prokaryotic and eukaryotic organisms.

1.1.1 Significance of the project

The rapid development and spread of resistance and the emergence of multi-drug resistant pathogens has created a pressing need for new classes of antimicrobial agents attacking novel microbial targets that will not be affected by existing resistance mechanisms. This need has coincided with a decline in the development of anti-

infective agents by pharmaceutical companies in favour of drugs treating chronic conditions such as diabetes, heart disease, neurodegenerative and inflammatory diseases. As a result the number of new anti-infective drugs brought to market in the last 20 years has been very low and there are currently relatively few in clinical development (Gringauz, 1997). Targeted discovery of anti-infective drugs requires identification of sufficiently selective microbial targets. Identification of new targets in microorganisms, particularly bacteria, is thus of great significance for the design or selection of compounds for clinical development (Section 1.3.2).

Many drugs have been successfully used therapeutically without knowledge of their mechanism of action (MOA) (Denisenko *et al.*, 27 Dec. 2002 International patent). However, an understanding of mechanism(s) of action is highly desirable in understanding the interaction of the pathogen with both the chemical and the host and assisting in the design of improved antimicrobials, determining effective combinations of drugs and understanding microbial resistance.

The mechanisms of action of developmental compounds that have not originated from target-based discovery should be explored as early as possible because such knowledge can aid in the modification of the drug scaffold for improved selectivity of action and an improved pharmaceutical profile. BDM-I is in commercial development as an antimicrobial agent (Section 1.2).

1.1.2 Plan of investigation for mechanism studies

Mechanism studies for a new agent are necessarily very open and involve many different types of investigation from enzymic assays to genetic analysis and observation of ultrastructure. The starting points and sequences of investigation vary greatly according to the existing biological information, the characteristics of the compound and the nature of the target organisms. Findings from early investigations

therefore direct future investigations. Many experiments presented here were conducted concurrently and others were constrained by availability of laboratory resources.

One problem facing investigators of antimicrobial mechanisms is to distinguish a primary activity from secondary and follow-on effects. Failure to distinguish these may erroneously suggest multiple mechanisms. Another is that many observations remain unexplained in the absence of detailed knowledge about microbial metabolic networks.

The plan of investigation for this characteristically open project was guided by:

i) review of the biological data available for BDM-I (Section 1.2) and related compounds (Section 1.2.1) for insights into likely (and unlikely) target pathways;

ii) further characterisation of the metabolic effects of BDM-I on selected microbial species as suggested by these results;

iii) investigate the metabolic targets of the main classes of antibacterial compounds (because most new compounds have attacked various aspects of existing, well-known target pathways); and

iv) investigate biochemical, physiological and structural species-specific effects (for secondary targets).

It was the latter investigations in the later stages of the project that were most fruitful because attempts to explain these specific effects led to a review of cell signalling in bacteria and the formation of the hypothesis that BDM-I interferes with cell signalling by inhibition of bacterial tyrosine phosphatases, a mechanism that could explain both broad and specific effects.

1.1.3 Summary of chapter contents

Results are presented by grouping similar topics rather than in a logical order of investigation. The chapters should not be viewed as the sequential order of investigation although some results presented in Chapters 5 and those in Chapter 6 were begun in the last year of the project. Not all results are presented here but none omitted contradict these findings.

Chapter 2 reports on the effect of thiol-containing compounds on BDM-I activity *in vitro*, investigations of the kill kinetics of BDM-I, its interactions with major antibiotics with known mechanisms of action and the action of BDM-I on the obligate intracellular bacterial pathogen, *Chlamydia trachomatis*.

Chapter 3 reports the effect of BDM-I on the bacterial cell wall/envelope and the cytoplasmic membrane and on the ultrastructure of bacteria and yeast.

In Chapter 4, effects of BDM-I on several cell functions are reported: production of exopolysaccharides and pigments, induction of variant colonial morphology and inhibition of swarming motility. Attempts to relate some of these latter observations directed attention to cell signalling in bacteria as a possible target.

Chapter 5 investigates the effect of BDM-I on ATP production, cell-free protein synthesis and on human and bacterial protein tyrosine phosphatases (PTP's). BDM-I is shown by an enzymic assay to be an inhibitor of a human and a bacterial PTP. Inhibition of bacterial PTP functions is therefore proposed as a major MOA of BDM-I in bacteria (and by extension, eukaryotic microorganisms). All earlier observations were reviewed for their consistency with this hypothesis.

A pilot DNA microarray analysis to investigate the effect of BDM–I on whole genome transcription in *Bacillus subtilis* is presented in Chapter 6.

1.2 BDM-I

BDM-I (3,4-methylenedioxy-β-nitropropene) belongs to the class of benzyl nitroalkenes (Figure 1-1). It is a yellow crystalline solid (MW 207.2), insoluble in water and soluble in organic solvents such as dimethylsulfoxide, ethanol and acetone, forming a solution of neutral pH. It has an octanol-water partition coefficient (K_D) of 345, a melting point range of 96-98°C, is stable to heat and labile to UV light on continued exposure. It binds strongly and reversibly to human serum albumin.

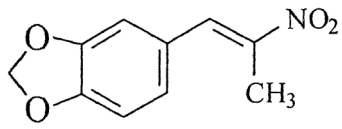


Figure 1-1. Molecular structure of BDM-I.

BDM-I has been shown to be effective *in vitro* against a wide range of Gram-positive and Gram-negative bacteria, filamentous fungi and yeasts (Table 1-1). BDM-I also has very similar activity against species within a genus where multiple species have been tested (*Staphylococcus*, *Enterococcus*, *Clostridium*, *Bacillus*, *Campylobacter*, *Proteus*, *Neisseria, Candida, Aspergillus* and *Rhizopus*) with minimum inhibitory concentrations (MIC's) rarely differing more than four-fold. BDM-I is equally active against clinical bacterial strains of major pathogens showing resistance to antibiotics, including methicillin resistant *Staphylococcus aureus* (MRSA) and vancomycin resistant enterococci (VRE), indicating an absence of resistance mechanisms to BDM-I (Denisenko *et al.*, 27 Dec. 2002 International patent, Nicoletti et al., unpublished).

Table 1-1. Minimum Inhibitory Concentration (MIC) of BDM-I (μg/mL) against representative bacteria and fungi.

Bacteria			
Gram-negative		**Gram-positive**	
Haemophilus influenzae RMIT 140/1-1	0.3	*Streptococcus pyogenes* RMIT 345/1	2
Pasteurella multocida RMIT 284/1-2	2	*Clostridium perfringens* NCTC 8237	2
Neisseria gonorrhoeae RMIT 240/2	5	*Staphylococcus aureus* ATCC 29213	6
Moraxella catarrhalis RMIT 211/2	16	*Corynebacterium xerosis* RMIT 53/5	8
Bacteroides fragilis RMIT 31/1	20	*Enterococcus faecalis* ATCC 29212	1b
Brucella abortus RMIT 33/1	64	*Lactobacillus casei* RMIT 190/3	64
Proteus vulgaris ATCC 13395	8	**Fungi**	
Yersinia enterocolitica RMIT 430/1	16	*Trichophyton rubrum* RMIT 235	1
Proteus mirabilis RMIT 281/1	128	*Pseudoallescheria boydii* RMIT 141	2
Acinetobacter calcoaceticus RMIT 3131	128	*Fusarium graminearum* RMIT 790/1	2
Salmonella Typhimurium ATCC 14028	304	*Aspergillus fumigatus* RMIT 702/1-1	4
Escherichia coli ATCC 25922	406	*Rhizopus stolonifer* RMIT 905/2-1	4
Klebsiella pneumoniae ATCC 13833	512	*Rhodotorula rubra* RMIT 655	8
Pseudomonas aeruginosa ATCC 27853	>512	*Candida albicans* ATCC 10231	8

Assays performed using standard methods outlined by the NCCLS.
(Denisenko *et al.*, 27 Dec. 2002 International patent, Nicoletti *et al.*, unpublished)

BDM-I has also demonstrated anti-protozoal activity. It is rapidly microbicidal to *Trichomonas vaginalis* at 2 μg/mL. BDM-I is also active against the intracellular sporozoan pathogens, *Plasmodium* and *Eimeria* which are discussed in Chapter 2.

BDM-I shows very low potential to produce resistant phenotypes. On long term exposure for 12-16 weeks BDM-I did not induce resistance *in vitro* in *Candida albicans*, fungi (*R. rubra*, *A. fumigatus*, *R. stolonifer*, *F. graminearum*) or Gram-positive and Gram-negative pathogens known to develop multi-drug resistance (*S. aureus* MRSA, *E. faecalis,* VRE, *C. perfringens*, *K. oxytoca*, *P. vulgaris*) (Brian *et al.*, 1946; Dominguez *et al.*, 1953; Milhazes *et al.*, 2006; Schales & Graefe, 1952; Worthen & Bond, 1970; Zatula, 1974). The failure to readily develop resistant phenotypes is advantageous for BDM-I as a putative anti-infective drug. However, lack of resistant

strains precluded the study of such phenotypes to identify metabolic or genetic changes associated with the mechanisms of action of BDM-I.

The development of other compounds in the same structural series as BDM-I will be facilitated by knowledge of its MOA, allowing more directed lead optimisation to generate more clinically useful compounds. Over 31 compounds based on the structure of BDM-I have been synthesized, chemically characterized and assayed against a panel of bacteria and fungi by whole cell assay (Nicoletti *et al.*, unpublished). Analogues were also compared for toxicity to zebrafish and with respect to octanol-water partition coefficients (K_D). BDM-I was found to be, overall, the most broadly active agent, although some analogues also showed broad and high activity against particular groups, including the enteric and related Gram-negative bacteria. Toxicity to zebrafish eggs generally correlated with toxicity to microorganisms. No gross effects on embryonic development of zebrafish were observed for BDM-I and selected analogues.

BDM-I is well tolerated in rodents and chicks on oral dosing (Nicoletti *et al.*, unpublished). In studies conducted in rats under good laboratory practice (GLP) conditions, the maximum tolerated single dose was 2000 mg/kg and a 7 day repeat dose of 300 mg/kg gave a no-observed adverse effect level (NOAEL) and a lowest lethal dose of <1150 mg/kg. BDM-I is poorly absorbed with 1-2% of the ingested dose reaching the blood. In non-GLP studies, a 7 day repeat oral dose of 200 mg/kg in canola oil was well tolerated by mice. A 28 day repeat oral dose study in broiler chicks gave a NOAEL of 125 mg/kg with some toxicity evident at 150 mg/kg. Intra-peritoneal doses of 10, 25 and 40 mg/kg BDM-I daily for 17 days were well tolerated in mice. Intravenous doses from 10 to 25 mg/kg given twice weekly for 3 weeks to nude mice were well tolerated with no adverse symptoms.

This relative lack of toxicity for such a broadly active antimicrobial compound suggests that the metabolic target of BDM-I in prokaryotic and eukaryotic microorganisms is sufficiently selective to allow for differential toxicity between microbial and mammalian cells.

1.2.1 Benzyl nitroalkenes

Substituted benzyl nitroalkene compounds have been studied previously for a number of interesting properties, including as broad spectrum antimicrobial agents. The antimicrobial properties of several benzyl nitroethenes have been demonstrated in bacteria (1952). Arylnitroalkene compounds prepared by Schales & Graefe (Cavier *et al.*, 1978; Worthen & Bond, 1970; Zatula, 1974) were found to be more effective against Gram-positive than Gram-negative bacteria and to have low oral toxicity to mice. With an increasing number of discoveries of conventional antibiotics, interest in this class of synthetic compounds subsided until the 1970's when several derivatives were shown to have broad anti-bacterial, anti-fungal and anti-protozoal activity (Milhazes *et al.*, 2006). Most recently, a structure-property-activity relationship study investigated the antibacterial activity of a number of benzyl nitroalkenes (Carter *et al.*, 2002; Dore & Viel, 1975; Kaap *et al.*, 2003; Kim *et al.*, 2003; Leite *et al.*, 2004; Micale *et al.*, 2003; Milhazes *et al.*, 2006; Ramos *et al.*, 1997). There has been recent interest in substituted benzyl nitroalkenes for their chemotherapeutic potential as anti-tumour agents (Leite *et al.*, 2004). Benzodioxoles, such as safrole and myristicin, which occur in a variety of plants, have been shown to have similar biological activities (Leite *et al.*, 2004). The benzodioxole derivatives reported have not included substitution with nitroalkenes (as for BDM-I). A series of benzodioxole-peptide derivatives did not exhibit antibacterial activity but showed anti-tumour activity for mouse sarcoma (Micale *et al.*, 2002; Micale *et al.*, 2003; Yokota *et al.*, 2000). Other benzodioxole

derivatives have been shown to have activity against a range of human tumours, *in vitro* and *in vivo* (Batra *et al.*, 1985; Jurd *et al.*, 1987) and to bind tubulin and to be anti-mitotic (Schwarz & Kehrenberg, 2006).

1.3 THE NEED FOR NEW ANTIMICROBIAL AGENTS

Antimicrobial compounds have provided an important means of defence against pathogenic microorganisms. The introduction of modern antibiotics into clinical practice was a significant medical advance, enabling successful treatment of feared and lethal bacterial diseases. Their widespread use has led to the rapid development and spread of resistant phenotypes and the emergence of multi-drug resistant pathogens which have become a major cause of therapy failure in infectious diseases. Without new antibiotics, rather than places of safe refuge for the sick, hospitals become dangerous reservoirs of resistant microorganisms making surgical procedures and clinical therapy precarious.

Protection from microbial pathogens relies on our ability to effectively manage current antimicrobial agents and continue to develop new classes of compounds. Modified versions of existing drug structures have proved cost-effective but quickly lose efficacy as bacteria acquire resistance with increasing rapidity (Projan, 2007). Opportunistic infections in immunocompromised patients, especially fungal infections due to *Candida* spp. and *Aspergillus* spp., have few successful treatments available and infection relapses are common. The problem extends to protozoal infections such as malaria (*Plasmodium* spp.) that also face a lack of chemotherapeutic options and the development of resistant strains. Microbial resistance is thus eroding the efficacy of current therapies and increasing the demand for new classes of anti-infective drugs attacking novel microbial targets.

Resistance has now developed to every major class of anti-infective drug in current use. Antibiotics vary in their ability to promote the development of resistant strains which may be related to their molecular structure and/or MOA (Nordmann *et al.*, 2007). The propensity to induce low levels of resistant phenotypes is a very desirable characteristic in new drugs.

The potential of an organism to develop resistance is influenced by the MOA of the drug, the metabolic capability of the organism, bacterial efficiency in exchanging genetic information and the availability of a pool of resistance genes. Multi-drug resistance has emerged in a number of significant and highly adaptive pathogens including *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Enterococcus* spp. and the enteric and related Gram-negative bacteria (El'Garch *et al.*, 2007). Transporter efflux pumps that render bacteria resistant to multiple unrelated drugs are widespread (Livermore & Woodford, 2006; Queenan & Bush, 2007). Production of extended-spectrum β-lactamases and carbapenemases by multi-drug resistant enteric Gram-negative bacteria makes them a clinical threat as many β-lactam antibiotics become ineffective (Blower & Supervie, 2007; Lambert, 2002; Musser, 1995; Smith, 2007). Many strains of *Mycobacterium tuberculosis* have become resistant to current therapies (Russell, 1998).

1.3.1 Therapeutic anti-infective agents

There are many therapeutic antimicrobial agents available today, mostly secondary metabolites of bacteria or fungi or semi-synthetic derivatives of these natural products. The major classes of anti-bacterial therapeutic agents are the naturally derived β-lactams, aminoglycosides, tetracyclines, macrolides, lincosamides and glycopeptides and the synthetic sulfonamides and fluoroquinolones(Denyer & Hugo, 1991; Denyer & Stewart, 1998).

These classes have provided a chemical platform for the successful synthesis of generations of drugs with improved stability, pharmacokinetics and spectrum of activity. The main sites of action for antibacterial drug classes are cell wall biosynthesis, protein synthesis, DNA replication and folate metabolism. The majority of antibiotics also have metabolic targets additional to the major targets.

Antiseptics

Antiseptics are synthetic compounds (eg. cationic compounds, phenolics and dyes) which generally have a much broader range of activity and display less selective toxicity than therapeutic antimicrobials and more than disinfectants and industrial biocides (Heath *et al.*, 1999). They generally target several structures or functions essential for viability and common to many microbial types and are rapidly microbicidal. Antiseptics are used at high concentrations where they exert non-specific effects and development of resistance by microorganisms is not a problem. Many target the cytoplasmic membrane and general cytoplasmic proteins while some have more specific effects, observable at low concentrations. Triclosan, a bisphenol is a broad spectrum biocide which inhibits FabI, an enzyme involved in chain elongation in bacterial fatty acid biosynthesis (Ioannou *et al.*, 2007; Kuyyakanond & Quesnel, 1992; Schweizer, 2001). Fatty acid biosynthesis is a recently identified novel target for development of anti-infective agents. Cationic agents such as chlorhexidine and quaternary ammonium compounds disrupt cell membranes causing leakage of intracellular constituents (Chu *et al.*, 1996).

There are, however, synthetic compounds that fall between the classification of a therapeutic anti-infective and an antiseptic because they display antimicrobial activity against a broad range of organisms and, while generally being relatively more toxic than commonly used therapeutic drugs, are less toxic than antiseptics and have

attributes that would allow selected therapeutic uses. BDM-I and the related benzyl nitropropenes are such a class of compounds.

1.3.2 Development of anti-infective drugs

The successful use of anti-infective drugs unfortunately seems to lead to an inevitable increase in resistant microorganisms and a loss of clinical efficacy which, in turn, drives the need to develop new anti-infective drugs. Unlike successful therapeutic drugs used in other areas, a continuing supply of novel antimicrobial agents is therefore needed to maintain effective therapy of infectious disease. Very few major new classes of therapeutic antimicrobial agents have been introduced since the azoles and fluoroquinolones, first approved in the 1960's. Semisynthetic derivatives optimised for the common targets and with special properties, including the circumventing of resistance mechanisms, continue to be the main source of new drugs.

Approaches to developing new antimicrobial agents include the classical screening of natural or synthetic compounds, structural changes to existing agents based on structure-activity relationships and rational design or searching the genome for novel targets against which compounds may be effectively designed or compound libraries screened (McDevitt & Rosenberg, 2001; Root *et al.*, 2003). Lead compounds may then be optimised for activity and pharmaceutical properties to obtain the most suitable derivative. In the last 15 years genomically-based targeted discovery of therapeutic drugs by high throughput screening of synthetic and natural chemical libraries using specific enzyme and cell-based assays has been used to identify compounds affecting an identified target(Chopra *et al.*, 2002). This approach however, has not yet been successful with anti-infective drugs as there has been a lack of sufficiently selective, new microbial targets. Exploitation of the well-understood microbial pathways affected by the major classes of antibiotics can identify further targets and new agents (Table

1-2) (Projan, 2007). However, new metabolic targets for which there are no resistance phenotypes are an urgent need.

The most successful approach for anti-infective drugs continues to be the traditional approach of assaying natural or synthetic compounds for inhibition using whole cell systems, characterizing the activity and then identifying cellular mechanisms responsible for the antimicrobial activity.

New approved drugs

The newer approved drugs tend to have a narrow spectrum of activity with a focus on targeting antibiotic resistant Gram-positive cocci. Very few new agents are directed at the multi-drug resistant Gram-negative bacteria. In the past 8 years, only six new agents have been approved for clinical use in humans – Telithromycin, Gemifloxacin, Daptomycin, Tigecycline, Linezolid and quinupristin/dalfopristin (Table 1-2). Of these, only Tigecycline is active against Gram-negative bacteria , highlighting the need for a greater focus on this group (Hancock, 2005; Nordmann *et al.*, 2007). Five of the six new drugs attack two existing bacterial targets, protein synthesis or DNA replication, with an inherent likelihood of eventual obsolescence with the emergence of resistance (Alborn *et al.*, 1991; Canepari *et al.*, 1990).

Only two, linezolid and daptomycin, belong to new structural classes. Only one of these has a truly novel target. Daptomycin, a narrow spectrum semi-synthetic cyclic lipopeptide, causes calcium-dependent depolarisation of the cell membrane, disrupting its energisation and interfering with multiple membrane functions, resulting in many secondary effects (Seedat *et al.*, 2006; Skiest, 2006). It is, as expected, rapidly bactericidal and to date no microbial mechanism of resistance has been identified. Linezolid, an oxazolidinone, is the only synthetic compound. It inhibits protein synthesis in a novel way by binding to the 50S sub unit and blocking assembly of the

Table 1-2. Recently approved anti-bacterial drugs.

Antimicrobial class	Antimicrobial compound (Trade name)	Approval date	Site of antimicrobial action / target	Spectrum	References
Streptogramin Cyclic peptides	quinupristin& dalfopristin combination (Synercid)	1999	50s Ribosome unit Blocks peptidyl transferase and promotes premature release of peptidyl chain	Gram-positive bacteria Legionella Chlamydia	(Berisio *et al.*, 2003; Farrell *et al.*, 2004)
Macrolide (ketolide)	Telithromycin (Ketek)	2001	Ribosome Blocks exit of peptide from 50S sub-unit	Gram-positive bacteria (MDR) Some Gram-negative bacteria	(Saravolatz & Leggett, 2003)
Fluoroquinolone	Gemifloxacin (Factive)	2003	DNA replication Bind to topoisomerases I & II	Gram-positive bacteria Anaerobes Some Gram-negative bacteria	(Alborn *et al.*, 1991; Canepari *et al.*, 1990)
Cyclic lipopeptide	Daptomycin (Cubicin)	2003	Cell membrane depolarisation	Gram-positive bacteria	(Olson *et al.*, 2006; Sum & Petersen, 1999)
Tetracycline (Glycylcycline)	Tigecycline (Tygacil)	2005	Ribosome Binds to 30S rRNA blocking binding of tRNA	Gram-positive and Gram-negative bacteria	(Jones *et al.*, 1996; Lin *et al.*, 1997; Swaney *et al.*, 1998; Zurenko *et al.*, 1996)
Oxazolidinone	Linezolid (Linezolid)	2007	Ribosome Interferes with tRNA binding to 23S rRNA preventing formation of 70S initiation complex	Gram-positive cocci. Clostridia and some Gram-negative	(Hunter *et al.*, 2004; Kennelly, 2002; Pawson & Scott, 2005; Tonks, 2006).

70S initiation complex, preventing translation of mRNA. Both compounds have a low spontaneous resistance rate but some anecdotal resistance has been reported in staphylococcal and enterococcal infections (Allen & Nicas, 2003; Appelbaum & Jacobs, 2005).

Drugs in development are predominantly modifications of existing scaffolds acting on existing targets and directed against Gram-positive bacteria. For example, dalbavancin and oritavancin are cyclic glycopeptides inhibiting cell wall biosynthesis and targeting the resistant Gram-positive cocci (Walker *et al.*, 2005). Ramoplanin, a lipoglycodepsipeptide antibiotic, also inhibits peptidoglycan biosynthesis and is very active against resistant Gram-positive bacteria (Fass, 1991).

1.3.3 Novel cellular targets for drugs

Protein kinases and recently phosphatases, are new targets in the development of therapeutic drugs for a range of chronic diseases in humans. They have not, to date, been proposed as suitably selective drug targets in microorganisms, eukaryotic or prokaryotic.

Protein phosphatases

Post-translational modification of proteins by the covalent addition and removal of phosphoryl groups is a major mechanism controlling intracellular signalling. Coordinated and complementary phosphorylation by protein kinases (PK's) and dephosphorylation by protein phosphatases (PP's), directly regulate molecular processes in response to extracellular and intracellular signals (Tonks, 2006; Zhang, 2002). PK's catalyse the formation in proteins of serine, threonine or tyrosine monoesters which are readily hydrolysed to the amino acid residue and free phosphate by cognate PP's.

There are two major classes of protein phosphatases in eukaryotes, those dephosphorylating serine and threonine (PSTP) and those dephosphorylating tyrosine (PTP). Most phosphorylation in eukaryotic cells occurs on serine and threonine residues. Tyrosine phosphorylation, although minor in signalling pathways in eukaryotes, controls normal cellular growth, progression through the cell cycle, cell differentiation, cell-to-cell communication, cell migration, gene transcription, apoptosis decisions and the immune response (2006).

Protein tyrosine phosphatases form a large family with a highly conserved catalytic site which is defined by its amino acid sequence (PTP signature motif) and in which cysteine and arginine are key to activity. The PTP family includes the tyrosine specific and dual specific phosphatases (DSP). The latter are less well conserved and

have little sequence similarity beyond the signature motif. DSP's are able to hydrolyse phosphate residues on tyrosine and either a serine or threonine on the same protein. Tonks (Grangeasse *et al.*, 2007; Kennelly, 2002; Ponting *et al.*, 1999; Shi *et al.*, 1998; Vincent *et al.*, 1999) reviews the structure, functions and regulation of PTP's and their role in signal transduction and as an underlying cause of human disease.

Bacterial phosphatases

Protein phosphorylation on tyrosine, serine and threonine was long considered a post-translational modification specific to eukaryotes. In prokaryotes the presence of protein tyrosine kinase and phosphatase activity was discovered much later. Homology searches across many bacterial species show a general distribution of orthologs for PK's and PP's (Grangeasse *et al.*, 2007). Tyrosine residues are less abundant than serine and threonine and more stable than histidine/aspartate residues. Tyrosine phosphorylation seems to regulate particular functions in bacteria different from those in eukaryotes (Grangeasse *et al.*, 2007; Kennelly, 2002). Bacterial phosphorylation is a rapidly expanding field of investigation and tyrosine phosphorylated proteins have been reported in a variety of bacteria (Kennelly, 2002). Bacteria encode a smaller number of PTP's compared to eukaryotic cells (Grangeasse *et al.*, 2007; Kennelly, 2002; Mijakovic *et al.*, 2003; Mijakovic *et al.*, 2005b; Morona *et al.*, 2002; Musumeci *et al.*, 2005; Vincent *et al.*, 1999; Vincent *et al.*, 2000). For many PTP genes the endogenous substrates have not yet been identified and their biological functions are unknown.

While bacterial PTP's have sequence and structure similarity to eukaryotic counterparts, those so far investigated are not as specialized as eukaryotic PTP's, showing functional overlap, greater catalytic versatility and a heterogeneous distribution. Most PTP's identified in bacteria are dual specific phosphatases, which are

more common in bacteria than in eukaryotes, or homologues of eukaryotic low molecular weight PTP's (LMWPTP's) (Grangeasse *et al.*, 1997; Grangeasse *et al.*, 1998; Musumeci *et al.*, 2005). LMW PTP's are the most ancient and highly conserved family of PTP's and show a high degree of identity with human homologs (Grangeasse *et al.*, 2007).

Tyrosine phosphorylation influences gene regulation and enzyme activity in bacteria and seems to be related to the virulence mechanisms of many pathogens (Musumeci *et al.*, 2005) and response to stresses which enable survival in hostile environment (Denisenko *et al.*, 27 Dec. 2002 International patent). To date there is no report of the development of antimicrobial agents targeting bacterial phosphatases.

CHAPTER 2 ANTIBACTERIAL ACTIVITY OF BDM-I

2.1 INTRODUCTION

BDM-I has been assayed for bacteriostatic and bacteriocidal activity by standard whole cell growth inhibition assays against a wide range of bacteria and fungi selected to represent broad taxonomic and metabolic types and to include clinically significant pathogens, particularly those exhibiting considerable development of resistance to current antimicrobial drugs (Denisenko *et al.*, 27 Dec. 2002 International patent). Representative species assayed previously are shown in Table 1-1.

Bacterial groups were differentiated on the basis of cell wall structure, which influences susceptibility to antimicrobial agents. These groups were Gram-positive bacteria, non-enteric Gram-negative bacteria, and enteric and related Gram-negative bacteria. Gram-positive bacteria included strictly aerobic, facultatively anaerobic, aerotolerant fermentative and anaerobic fermentative species of clinical significance. Non-enteric Gram-negative bacteria included nutritionally and environmentally fastidious and parasitic bacteria (aerobic, facultative and anaerobic) that are relatively susceptible to antibiotic drugs. Enteric Gram-negative bacteria and related genera are those characterised by a greater resistance to drugs and the ready development of multi-drug resistant phenotypes.

BDM-I is broadly but variably antibacterial, with greatest activity overall against Gram-positive bacteria and least against enteric and related Gram-negative rods. It is highly active against fastidious and parasitic Gram-negative rods and cocci, with *Neisseria* and *Haemophilus* being among the most susceptible bacteria tested (Denisenko *et al.*, 27 Dec. 2002 International patent). BDM-I also shows very consistent whole cell growth inhibition against different species within a genus.

Multiple species within *Staphylococcus*, *Enterococcus*, *Clostridium*, *Bacillus*, *Campylobacter*, *Proteus*, *Neisseria, Candida, Aspergillus* and *Rhizopus* have MIC values rarely differing more than four-fold. BDM-I is slowly bactericidal to the majority of genera tested, with the Minimum Microbicidal Concentration (MMC) usually the same or two-fold higher than the MIC. BDM-I is also highly and uniformly active against a broad range of unicellular and filamentous fungi from different taxonomic phyla and is fungicidal to all species assayed (NCCLS, 2002; NCCLS, 2003).

Obligate parasites have reduced structural, physiological and metabolic activities, using host metabolism to fulfil some essential metabolic functions. BDM-I has been shown to inhibit the replication of *Plasmodium falciparum,* a sporozoan parasite causing malaria. BDM-I inhibited trophozoite growth and replication in red blood cells in a concentration-dependent manner at blood levels achievable and tolerable in mice (Nicoletti *et al.*, unpublished). Activity against four species of *Eimeria* commonly causing coccidiodosis in poultry was shown in a chick model of infection (Nicoletti *et al.*, unpublished). Oral treatment with BDM-I reduced levels of infection in chicks challenged with virulent *Eimeria* spp. and reduced oocyst counts in infected chicks in a dose-dependent manner. Activity against such pathogens indicates intracellular activity and restricts the possible targets to those in pathogen or host cells that are essential for pathogen infectivity and replication.

The generally uniform activity of BDM-I against microorganisms using oxidative, fermentative and facultative energy metabolism and under aerobic and anaerobic atmospheric conditions, suggests that antimicrobial activity is not targeted to cellular energy pathways. The activity of BDM-I across prokaryotic and eukaryotic genera suggests that it is acting on one or more highly conserved cellular target(s). Such target(s) appear to be heterogeneously distributed across bacterial species. The

more uniform inhibition across fungal genera suggests that the target(s) in fungi are more highly conserved, functionally similar and more evenly distributed across taxonomic types. The unusually broad spectrum of activity is more characteristic of a disinfectant or antiseptic than a therapeutic antimicrobial agent. However, BDM-I is not uniformly rapidly microbicidal as are biocides, suggesting the toxic mechanisms are more selective.

2.2 ACTIVITY SPECTRUM FOR BDM-I: MINIMUM INHIBITORY AND MINIMUM MICROBICIDAL CONCENTRATIONS

2.2.1 Background

An important consideration in investigations of mechanisms of action is the selection of appropriate bacterial species, agent concentration ranges and assay conditions to reveal a metabolic effect. Failure to optimise an assay may result in a failure to demonstrate an effect. As an extension of the activity spectrum of BDM-I, a variety of bacteria were investigated based on their susceptibility to BDM-I and suitability for assays designed to investigate the MOA of BDM-I. The yeast, *Candida albicans* was also included in assays to provide a comparison of bacteria with a unicellular eukaryote. The MIC and MMC of BDM-I was determined in relevant media to provide a basis for selection of concentrations and time frames for further investigations.

2.2.2 Materials and Methods

Microbial stocks, media and test compounds

Strains are as follows: *Bacillus cereus* RMIT 30/7, *Bacillus subtilis* ATCC 6633, *Enterococcus faecalis* ATCC 29212, *Staphylococcus aureus* ATCC 29213, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 13883, *Moraxella catarrhalis* RMIT 211/2, *Proteus mirabilis* RMIT 281/1-4, *Proteus vulgaris* ATCC 13315, *Pseudomonas aeruginosa* ATCC 27853, *Serratia marcescens* RMIT 342/1-32 and *Candida albicans* ATCC 10231.

Stocks of all microorganisms were kept in -80°C in MHB (Oxoid, Cambridge, UK) with 20% v/v glycerol (BDH chemicals, Poole, UK). All strains were subcultured onto Nutrient agar (NA, Oxoid) and subcultures between three and nine used to prepare inocula. Inoculum densities were prepared by adjusting a suspension to match McF 0.5 turbidity standard (equal to ~1.5×10^8 bacterial cells/mL or ~1.5×10^6 yeast cells/mL) and subsequent dilution to the required density.

Antibiotics, erythromycin and tetracycline (Sigma-Aldrich, St-Louis, MO, USA) and ciprofloxacin and vancomycin (ICN Biomedicals; now MP Biomedicals, Irvine, CA, USA) were received as dry powders, weighed, dissolved in diluent and stored as per manufacturer's instructions. BDM-I was dissolved at 100 mg/mL concentrated stock solution in dimethyl sulfoxide (DMSO, #103234, AnalaR®, BDH Chemicals) and stored at -20°C. Aliquots and dilutions of BDM-I were stored in the dark at room temperature for up to 1 month and retained inhibitory potency when assayed by measurement of the MIC in bacterial species. The highest concentration assayed was 512 μg/mL for species able to grow well in 1% v/v DMSO.

Minimum Inhibitory Concentrations

Broth microdilution testing was based on the National Committee for Clinical Laboratory Standards standard methods (NCCLS, 2002; NCCLS, 2003) in MHB (Oxoid) or Sabouraud Liquid Medium (SLM, Oxoid) for *C. albicans*. Microplate assays were performed in clear, round-bottomed, 96-well plates (Sarstedt Australia, SA, AUS) with a total volume of 200 µL per well. Inoculum densities were estimated by suspension turbidity using McF0.5 standard. Standard inoculum densities were approximately 1×10^5 Colony Forming Units per mL (CFU/mL) for bacteria and 1×10^4 CFU/mL for *C. albicans*.

The MIC of BDM-I against bacterial strains used in assays was determined in relevant media to provide a basis for selection of suitable assay concentrations. Inoculum densities higher than the standard were required for some assays, therefore MIC's were determined for different inoculum densities and reported as MIC (MIC using the standard inoculum density) and MIC_H (MIC using a 10-fold higher inoculum).

Inoculum densities were confirmed by serial dilution plating onto NA and incubation aerobically at 37°C for 24 h. BDM-I was added to plates at 2× test concentrations in 100 µL media. Ciprofloxacin was used as an internal positive control. Microplates were incubated 18-24 h at 37°C aerobically before reading wells visually for turbidity. All assays included duplicated wells and were replicated at least twice. The geomean MIC (µg/mL) for each strain was adjusted to the nearest $\log_2$ dilution tested. MIC results were reported as MIC and MIC_H for standard and 10-fold higher inoculum densities respectively.

Minimum Microbicidal Concentrations

The minimum microbicidal concentration (MMC) for BDM-I was determined following the MIC assay. Wells without visible growth of the test organism were sampled (10 µL), plated onto NA and incubated 48 h at 37°C aerobically. Viable organisms were calculated as colony forming units per mL (CFU/mL). The MMC for each strain was defined as the minimum concentration sufficient to decrease the number of viable cells from the initial inoculum by 99.9% (equivalent to a 3-$\log_{10}$ reduction in CFU/mL on subculture to NA).

2.2.3 Results

The geometric mean MIC values (µg/mL) for the test strains of bacteria and *Candida albicans*, in MHB with standard or high inoculum densities are shown in Table 2-1. There was no significant increase in the MIC with a 10-fold higher inoculum (MIC_H). These values were used as the basis for choosing treatment concentrations in further investigations.

2.2.4 Discussion

The activity of BDM-I presented here, as measured by MIC and MMC, reflects that found in more extensive studies (Nicoletti, *et al*., unpublished). BDM-I is clearly acting in a selective manner with a range of MIC and MMC values obtained for even a small representative sample of bacteria and fungi. The differential effect on genera of a group, especially among Gram-Negative bacteria, has not yet been explained. It is possible that the activity of BDM-I could be affected by intracellular access and therefore connected to the composition or structure of bacterial membranes.

Table 2-1. Inhibitory and microbicidal activity of BDM-I against Gram-positive and Gram-negative bacteria and *Candida albicans* strains used in this study.

	MIC & MMC (µg/mL)[a]		
	MIC	MIC_H[b]	MMC[c]
Gram-positive bacteria			
Bacillus cereus RMIT 30/7	8	8	16
Bacillus subtilis ATCC6633	8	8	16
Enterococcus faecalis ATCC 29212	16	16	512
Staphylococcus aureus ATCC 29213[d]	8	16	>512
Gram-negative bacteria			
Escherichia coli ATCC 25922	512	>512	>512
Klebsiella pneumoniae ATCC 13883	256	>512	>512
Moraxella catarrhalis RMIT 211/2	8	8	8
Proteus mirabilis RMIT 281/1-4[e]	128	-	>512
Proteus vulgaris ATCC 13315	8	8	32
Pseudomonas aeruginosa ATCC 27853	>512	>512	>512
Serratia marcescens RMIT 342/1-32	>512	-	-
Yeast			
Candida albicans ATCC 10231	4	8	8

MIC assays performed using standard methods outlined by the NCCLS (Snyder & MacIntosh, 2000)

[a]The geometric mean MIC, MIC_H and MMC (µg/mL) for each strain was adjusted to the nearest $\log_2$ dilution tested.

[b]The MIC_H was determined using a 10-fold higher inoculum than for the MIC.

[c]The inoculum level used for the MMC was that used for the standard MIC. The MMC is the lowest concentration showing a 3 $\log_{10}$ reduction in CFU/mL from the initial inoculum density.

[d]The MIC for ciprofloxacin against *S. aureus* (geomean 0.3 µg/mL) was used as a positive control and was within the NCCLS guidelines acceptable range on all occasions.

[e]Due to the swarming by *P. mirabilis* plate counts to determine MMC were not performed.

2.3 ACTIVITY AGAINST AN LOS-DEFICIENT MUTANT

2.3.1 Background

Among MIC results, the low susceptibility of some enteric Gram-negative bacteria to BDM-I was of particular interest. Gram-negative bacteria are characterised by a phospholipid outer membrane (or envelope) which contains variable levels and types of hydrophilic liposaccharides in the outer leaflet. While the lipid A and core structures of these liposaccharides in the outer membrane (OM) remain similar between all Gram-negative bacteria, the repeat sugar units can be unique to a species or strain and together have antigenic properties. Enteric bacteria generally possess high density lipopolysaccharides (LPS) and a greater resistance to lipophilic drugs while non-enteric bacteria generally possess a lower density of lipo-oligosaccharide (LOS) with a shorter, non-repeating chain (O-antigen). These structures have antigenic properties and are virulence factors that provide an effective barrier to hydrophobic molecules, detergents and host proteins (Heinrichs *et al.*, 1998; Kanipes *et al.*, 2004). Mutants that lack LPS or LOS structures have been found to lose their inherent resistance to antibiotics due to major alterations in the OM and are described as having a deep rough phenotype (Perera *et al.*, 2007). BDM-I is a highly lipophilic molecule and its relative inactivity against many Gram-negative bacteria was hypothesised to be partly due to its exclusion by the OM.

To investigate whether the hydrophilic OM of Gram-negative bacteria was partly responsible for the low activity of BDM-I a *Campylobacter jejuni* mutant possessing the smallest possible LOS component (*WaaF*) was compared to the wild type strain (*C. jejuni* HB93-13) (Friedman *et al.*, 2000).

Campylobacter are fastidious microaerophilic animal pathogens which invade intestinal epithelial cells and are among the most common cause of bacterial

diarrhoeal disease worldwide (2006). BDM-I shows relatively low activity against *Campylobacter* spp. with MIC's from 64 to 256 µg/mL (Nicoletti *et al*., unpublished). The mutant C. *jejuni* strain and the similarity of structure of the Gram-negative envelope allows extrapolation of the findings to other Gram-negative bacteria.

2.3.2 Materials and Methods

Campylobacter jejuni HB93-13 and the WaaF mutant lacking lipo-oligosaccharides (LOS) were obtained from Dr Viraj Perera (RMIT) and stored in Brucella broth (BB, BD Biosciences, San Jose, CA USA) with 20% v/v glycerol at -80°C. *C. jejuni* HB93-13 and the mutant were grown for 48 h on Horse blood agar (HBA, Oxoid) with Campylobacter Growth Supplement (Skirrow's, Oxoid) at 42°C under microaerophilic conditions - 5% oxygen, 10% carbon dioxide and 85% nitrogen (CampyGen sachet, Oxoid). The test inoculum was prepared from an 18 h culture incubated at 42°C in BB with Campylobacter growth supplement. Inoculum densities were confirmed by viable plate counts on HBA for 48 h at 42°C under microaerophilic conditions. Assays were performed in sterile Wassermann vials in total volumes of 2 mL. Doubling dilutions of BB alone or with 2× BDM-I in 1 mL were prepared in each tube, inoculated with 1 mL volumes of *C. jejuni* suspension and incubated for 48 h at 42ºC under microaerophilic conditions. Tubes showing no turbid growth were plated onto HBA and incubated for 48 h at 42ºC under microaerophilic conditions to determine the minimum microbicidal concentration.

2.3.3 Results

The mean MIC's for BDM-I against the *C. jejuni* parent (HB9313) and LOS mutant (WaaF−) were each 256 µg/mL and the geometric mean MMC for both was 362 µg/mL. Possession of a severely truncated LOS structure in the WaaF mutant of *C.*

jejuni HB9313 did not result in a greater susceptibility to BDM-I indicating that the presence of intact LOS in the outer leaflet of the OM was not a barrier to entry of BDM-I in *Campylobacter*.

2.3.4 Discussion

The amount of LOS on the envelope of *C. jejuni* was shown not to be a factor in the low activity of BDM-I against *Campylobacter*. The similarity in structure and function of LOS and LPS side chains in the outer leaflet of the envelope in Gram-negative bacteria, supports the extrapolation of the absence of a barrier effect to LPS-containing enteric and related bacteria. BDM-I shows a considerable range in activity against enteric Gram-negatives with MIC values varying from 8 μg/mL for *Proteus vulgaris* to >512 μg/mL for *Pseudomonas aeruginosa*. A barrier effect is unlikely to explain this variability since the more susceptible enteric species have lower MIC values than bacteria which have a lower LPS content including *Brucella*, *Campylobacter* and *Bacteroides* (Table 1-1). It seems unlikely that the lipophilicity of BDM-I contributes significantly to its lack of activity against high LPS bacteria. Milhazes *et al*. (Denisenko *et al.*, 27 Dec. 2002 International patent) showed that the antibacterial activity of a series of benzyl nitroalkene derivatives against Gram-positive cocci was not correlated with lipophilicity but did not report testing of Gram-negative bacterial species.

2.4 EFFECT OF THIOL-CONTAINING COMPOUNDS ON INHIBITORY ACTIVITY

2.4.1 Background

Evaluation of how inhibitory activity of an agent is affected (positively or negatively) by growth conditions, stresses of the external environment or cellular metabolites, can provide clues to antimicrobial mechanism(s) of action.

External factors affecting the activity of an agent *in vitro* include temperature, pH, oxidation-reduction potential, ultra-violet light and the presence of interfering compounds. The antimicrobial activity of BDM-I has been found to be unaffected by a 4 $\log_{10}$ change in medium pH, by incubation in aerobic and anaerobic atmospheres, by changes in incubation temperature and to be minimally affected by the presence of 10% whole blood or plasma or DNA (0.05% w/v), the MIC generally differing no more than two-fold (Eyer, 1994). BDM-I binds strongly and reversibly to serum albumin. It is slowly degraded by exposure to UV light, losing 4-fold activity after exposure to natural light for 6 weeks (Nicoletti *et al*, unpublished).

This investigation extends these investigations and reports on the effects of the presence of thiol-containing reducing compounds, cysteine and dithiothreitol, on the antibacterial action of BDM-I. Thiol-containing compounds activate nitroheterocyclic antimicrobial agents such as the nitrofurans and nitroimidazoles by conversion to toxic nitrosamines by intracellular nitroreductases (Markowitz & Williams, 1985; Nakken *et al.*, 1960). Thiol compounds have been reported to interfere with the activity of penicillins and cephalosporins by opening of the β-lactam ring (2004).

Cysteine is a low-molecular weight thiol compound present in many structural and enzymic proteins. The thiol group is nucleophilic, usually in the thiolate

form (S^-) and readily oxidised to the disulfide cystine. It is for example a component of the cellular anti-oxidant, glutathione. Cysteine is of particular interest as the signature amino acid residue at the catalytic site of protein tyrosine phosphatases (Section 1.3.3 & Chapter 5). Dithiothreitol (1,4-bis-sulfanylbutane-2,3-diol) is used in biochemical reactions to reduce disulphide bonds to dithiols and to protect thiol groups.

2.4.2 Materials and Methods

Stock solutions (5 M) of cysteine (#C7880, Sigma-Aldrich) and dithiothreitol (DTT, Bio-Rad Laboratories, CA, USA) were prepared in sterile distilled water and diluted to 3× working stock solutions in MHB. Test concentrations were of cysteine and DTT were 0.1 and 1 mM. BDM-I test concentrations were 2-fold from 0.5 to 256 µg/mL. Strains used were *S. aureus* ATCC 29213, *E. faecalis* ATCC 29212, *B. cereus* RMIT 30/7 and *P. vulgaris* ATCC 13315. Assays were performed in 96-well microplates (Sarstedt Australia), which were prepared with 50 µL each of 3× BDM-I, 3× cysteine or 3× DTT and inoculated with 50 µL of bacterial inoculum. Microplates were incubated for 24 h at 37°C aerobically and MIC values recorded based on visual turbidity as for standard MIC assays (Section 2.2.2). Assays were performed at least twice and the geometric mean calculated.

2.4.3 Results

Molar excesses of 2-4 DTT and cysteine had no effect on the MIC for BDM-I against any of the species tested. Greater molar excesses of DTT and cysteine also failed to have a significant impact on the MIC for BDM-I against S. aureus and P. vulgaris. There was a significant change in the MIC for *E. faecalis* in the presence of a 90-fold excess of cysteine and 6-fold excess of DTT, and for *B. cereus* in the presence of a 10-fold excess of DTT.

Table 2-2 Effect of cysteine and dithiothreitol on the MIC for BDM-I.

Bacterium	GeoMean MIC for BDM-I (µg/mL)[a] in presence of:						
	MHB	**0.1 mM Cysteine**	**1 mM Cysteine**	**10 mM Cysteine**	**0.1 mM DTT[b]**	**1 mM DTT[b]**	**10 mM DTT**
Staphylococcus aureus ATCC 29213[c]	4 (0.02)	6 (0.03)	4 (0.02)	6 (0.03)	8 (0.04)	11 (0.05)	304 (1.47)
Enterococcus faecalis ATCC 29212	10 (0.05)	10 (0.05)	10 (0.05)	23 (0.11)	8 (0.04)	32 (0.15)	362 (1.75)
Bacillus cereus RMIT 30/7	5 (0.02)	5 (0.02)	7 (0.03)	10 (0.05)	6 (0.03)	21 (0.10)	362 (1.75)
Proteus vulgaris ATCC 13315	6 (0.03)	6 (0.03)	8 (0.04)	11 (0.05)	8 (0.04)	8 (0.04)	215 (1.04)

[a]BDM-I molar concentrations equivalent to the MIC shown in table in brackets.

[b]DTT – dithiothreitol.

[c]The MIC for ciprofloxacin was used as an internal control and acceptable according to NCCLS guidelines (0.25 µg/mL).

2.4.4 Discussion

Cysteine and DTT inhibited the action of BDM-I in a whole cell MIC assay against two of the four species tested. Against *E. faecalis*, according the the increase in MIC, DTT was twice as inhibitory as cysteine. This inhibition is likely to be due to thiol reduction of BDM-I to an inactive benzyl nitroalkane.

Park & Pei (Milhazes *et al.*, 2006) showed that some benzyl nitroalkenes (0.02 mM) reacted readily and reversibly with mercaptoethanol (1 mM), resulting in saturation of the double bond and a ~200-fold inactivation of their ability to inhibit some PTP's in an enzyme assay. They further proposed that the interaction with mercaptoethanol was different from the reaction occurring directly between benzyl nitroalkenes and cysteine residues at the PTP catalytic site that is responsible for inhibition of enzyme activity. The explanation that cysteine and DTT similarly interfere with the activity of BDM-I by reduction of the propene side chain is plausible if BDM-I acts by inhibition of tyrosine phosphatases. Inhibition of two PTP's in an enzymic assay is demonstrated in Chapter 5.

The ability of thiols to inhibit BDM-I by reduction suggests that the nitro group and/or the double bond is required for activity. The double bond has been shown recently to be essential to the antimicrobial activity of benzyl nitroalkenes (Tocher, 1997). The likelihood that cysteine and DTT are acting here as reducing agents is suggested by the two-fold greater inhibition of BDM-I against *E. faecalis* by DTT which has two thiol groups compared to one for cysteine. The lack of enhanced activity of BDM-I in the presence of thiol-containing compounds suggests BDM-I does not act in a similar manner to nitroheterocyclic antimicrobial agents where activity depends on the formation of an active amine by bacterial nitroreductases (Nikaido & Saier, 1992).

Cysteine and DTT may interact with BDM-I in the medium or inside the cell. As the peptidoglycan layer of the cell wall of Gram-positive bacteria is relatively porous, cysteine and DTT would pass through readily. This is evidenced here by the greater inhibitory effect of thiols in Gram-positive species. The lesser effect on the inhibitory action of BDM-I on *P. vulgaris* could be due to the presence of the envelope providing an additional membrane barrier to the entry of these small hydrophilic molecules. These results suggest that interaction between BDM-I and thiols is occurring intracellularly and not just in the medium.

Both the envelope and plasma membrane of Gram-negative bacteria contain transport proteins that mediate the passive and active transport of polar solutes (Gilson *et al.*, 1988). The Gram-negative envelope has some barrier activity and contains porins that allow the diffusion of small nutrients. Gram-negative bacteria transport sugars and amino acids by binding these substrates to periplasmic binding proteins. Similar binding proteins anchored in the plasma membrane have been described in Gram-positive bacteria (Higgins, 2001). Sugars and amino acids are transported across the cytoplasmic membrane of bacteria by a range of ABC (ATP-binding cassette) transporters specific to different nutrients (2004). Cysteine and DTT, which are

assumed to be transported similarly to sugars, will compete for transport binding proteins which will limit their intracellular concentration despite the molar excesses supplied in the medium. BDM-I is a small lipophilic molecule and should readily diffuse across microbial membranes. There was a low level of inhibition (2-4-fold) shown by a 6-90-fold molar excess of cysteine and DTT in the whole cell assay reported here, compared to a 200-fold inhibition by a 50-fold molar excess of mercaptoethanol in the enzyme assay reported by Park & Pei (Clifton *et al.*, 2004). This could be explained by the interaction of cysteine and DTT with complex medium constituents, their restricted transport into the cell and their interaction with cytoplasmic components.

2.5 *IN VITRO* ACTIVITY AGAINST *CHLAMYDIA TRACHOMATIS*

2.5.1 Background

*Chlamydi*a are among the most simple prokaryotic cells, lacking several specialised metabolic functions and relying mainly on host epithelial cells to supply energy and metabolites for replication. *Chlamydia* have a unique biphasic development cycle, existing as two distinct functional and morphological forms over the life cycle of 48-72h. Elementary bodies (EB's) are small, metabolically inactive, stable and adapted for extracellular survival. They are capable of infecting a variety of cell types but primarily enter and differentiate within epithelial cells. EB's bind to host cells and induce endocytosis forming intracellular vacuoles (inclusion bodies) (AbdelRahman & Belland, 2005). *Chlamydia* actively modify the inclusion membrane to incorporate host components to prevent fusion with endosomes or lysosomes and insert chlamydial proteins (AbdelRahman & Belland, 2005). Following endocytosis, within the inclusion body, EB's differentiate into larger reticulate bodies (RB's) within 6-8 h. RB's have an inner and OM like other Gram-negative bacteria and use aerobic respiration. The

metabolically active RB's replicate by repeated cycles of binary fission before redifferentiation into EB's which are released by cell lysis to continue the infection cycle (Stephens *et al.*, 1998).

Chlamydiae have a small genome and are phylogenetically distant from other eubacteria. Most of the 28% predicted proteins that are unique to chlamydiae have unidentified functions (Gupta & Griffiths, 2006; Stephens *et al.*, 1998). Many encoded proteins show only a low level of homology with known bacterial proteins. Those with identified or suggested functions are related mainly to the vacuole membrane, transport of nutrients and DNA repair, replication and translation (Stephens *et al.*, 1998).

Chlamydia are energy parasites and import ATP using chlamydial ADP/ATP translocases similar to those in *Rickettsia* spp. Genetic analysis, however, shows they possess genes for restricted oxidative and substrate level production of ATP and use glutamate as the primary carbon source, supplemented by glucose and glutarate. (2003). *Chlamydia* also import host amino acids, purines and pyrimidines despite possessing some genes capable of limited synthesis. Therefore *Chlamydia*, as obligate intracellular parasites with a clear development cycle and restricted metabolic capabilities, are an attractive organism in which to study possible mechanisms of action.

This study investigates the activity of BDM-I against *Chlamydia trachomatis,* which infects epithelial cells and is a prevalent cause of sexually transmitted disease and eye infections in humans. The effect of BDM-I on the infection of epithelial cells by EB's and the replication of RB's was assessed in a cell culture assay in McCoy cells infected with a clinical isolate of *C. trachomatis*.

2.5.2 Materials and Methods

McCoy cells and a clinical isolate of *Chlamydia* trachomatis were supplied and maintained by Dr. Robert Alexander (Virology Laboratory, Royal Children's Hospital, Melbourne, Victoria, Australia). Methods were based on general methods for Chlamydia cell assays and those used by Suchland *et al.* (Suchland *et al.*, 2003) and are described below. BDM-I was assayed for inhibition of binding and endocytosis of the elementary body and inhibition of reticulate body replication in infected cells.

Briefly, epithelial cell monolayers (McCoy) were infected and the chlamydial bodies detected by immunofluorescent staining. The level of infection of the monolayer was recorded manually under a fluorescent microscope (IX71, Olympus Australia, Vic, AUS). BDM-I was assayed to determine whether it had an inhibitory effect on infection of McCoy cells by EB's and whether it inhibited replication of RB's in inclusion vacuoles.

Monolayer preparation

McCoy cells (ATCC, CRL-1696), a mouse fibroblast line, were grown and maintained in growth media (Minimum Essential Medium with Earle's Salts, 7.5% w/v sodium bicarbonate, 200 mM glutamine, non-essential amino acids, 100 mM sodium pyruvate and 10% v/v foetal calf serum, Thermotrace, Vic., AUS). Cells were grown to confluent monolayer in a 175 cm^2 culture flask (Greiner Bio-One, Frickenhausen, Germany). Cells were washed with phosphate buffered saline, harvested with trypsin/EDTA at 37°C (2-5 min), resuspended in growth media and 200 µL used to seed each well of a 96-well tissue culture treated microplate (#9102, Corning®, NY, USA) with ~3×10^5 cells /mL). Plates were grown to confluent growth for approximately 72 h before infection with a clinical strain of *C. trachomatis*.

Chlamydia titration

Before assaying BDM-I for activity the *Chlamydia trachomatis* stock suspension was titrated to estimate the number of infectious units. A stock vial of *C. trachomatis* (clinical isolate) was recovered from liquid nitrogen storage and the contents diluted 10-fold to 10^{-8} in Maintenance Medium (MM) (growth medium with 0.5 µg/mL cycloheximide and 4.5 g/L glucose). The medium was aspirated from the 96-well microplate containing confluent McCoy cells and 100 µL of each dilution of *C. tracomatis* was added to triplicate wells. The plate was centrifuged for 50 min at 35°C at 340 ×g (Hettich® Rotanta 96R, Andreas Hettich & Co., Tuttlingen, Germany). The medium was aspirated, replaced with 200 µL MM and incubated 48 h, 37°C, 5% CO_2 before fixing cells and staining as described below.

Assay for inhibition of cell infection

A dilution of *C. trachomatis* in MM determined from the titration to give a suitable level of infection was used as the inoculum. Medium was aspirated from the plate of McCoy cells and 100 µL each of Chlamydia and BDM-I or tetracycline (positive control) added to appropriate wells. The plates were centrifuged at 35°C for 50 min at 340 ×g (Hettich® Rotanta 96R, Andreas Hettich & Co.) to adsorb the Chlamydia into the McCoy cells. The aspirated medium was replaced with 200 µL MM containing 1× BDM-I, or tetracycline (#T7660, Sigma-Aldrich) as a positive control and 100 µL MM to maintain test concentrations. A titration series was also included on each assay plate to verify the number of infectious units of *Chlamydia*. Plates were incubated at 37°C in 5% CO_2 for 48 h.

Fixation and staining

All cells were fixed and stained with fluorescein-conjugated monoclonal antibodies to *C. trachomatis* (Bio-Rad Laboratories) *in situ* at 48 h post-infection. The

medium was aspirated and the wells left to air-dry for 2 minutes. Cells were fixed at -20°C for 15 min in acetone:methanol (1:2) (BDH Chemicals). The fixative was aspirated and the cells left to air-dry for 3 min at room temperature. One drop of *C. trachomatis* monoclonal antibody (Bio-Rad Laboratories) was added to each well, the plate covered with parafilm to increase the humidity and incubated at 37°C in 5% CO_2 for 1 h. All wells were washed several times in immunofluorescence washing buffer (Appendix A), once in distilled water and left to dry for 2 min at room temperature. Mounting medium (Appendix A) was added to each well and a fluorescent microscope (IX71, Olympus Australia) used to view fluorescent antibody bound to the major outer membrane protein of elementary and reticulate bodies. The assay was completed once and photos were taken with a digital camera (A525, Canon Australia, NSW, AUS) using a microscope eyepiece attachment.

Assay for inhibition of *Chlamydia* post-infection

Drugs were tested against a chlamydial infection, established for 24 h using the method described above except that the addition of drugs was delayed until 24 h after infection. This assay was repeated twice and phots taken as above.

2.5.3 Results

BDM-I, when added at the time of inoculation of the monolayer with EB's, reduced the number of RB's in McCoy cells at 48 h compared to the untreated control. Photographs of the fluorescent chlamydial bodies in infected McCoy cells are shown in Figure 2-1. BDM-I at a concentration of 5 µg/mL gave a similar level of reduction of RB's to that of tetracycline at 0.06 µg/mL, indicative of an approximately 80× lower activity by concentration. Results for tetracycline were comparable to those reported in previous studies where, in the absence of standardised methodology, the MIC (0.25 µg/mL) was conservatively defined as 2-fold above the concentration resulting in

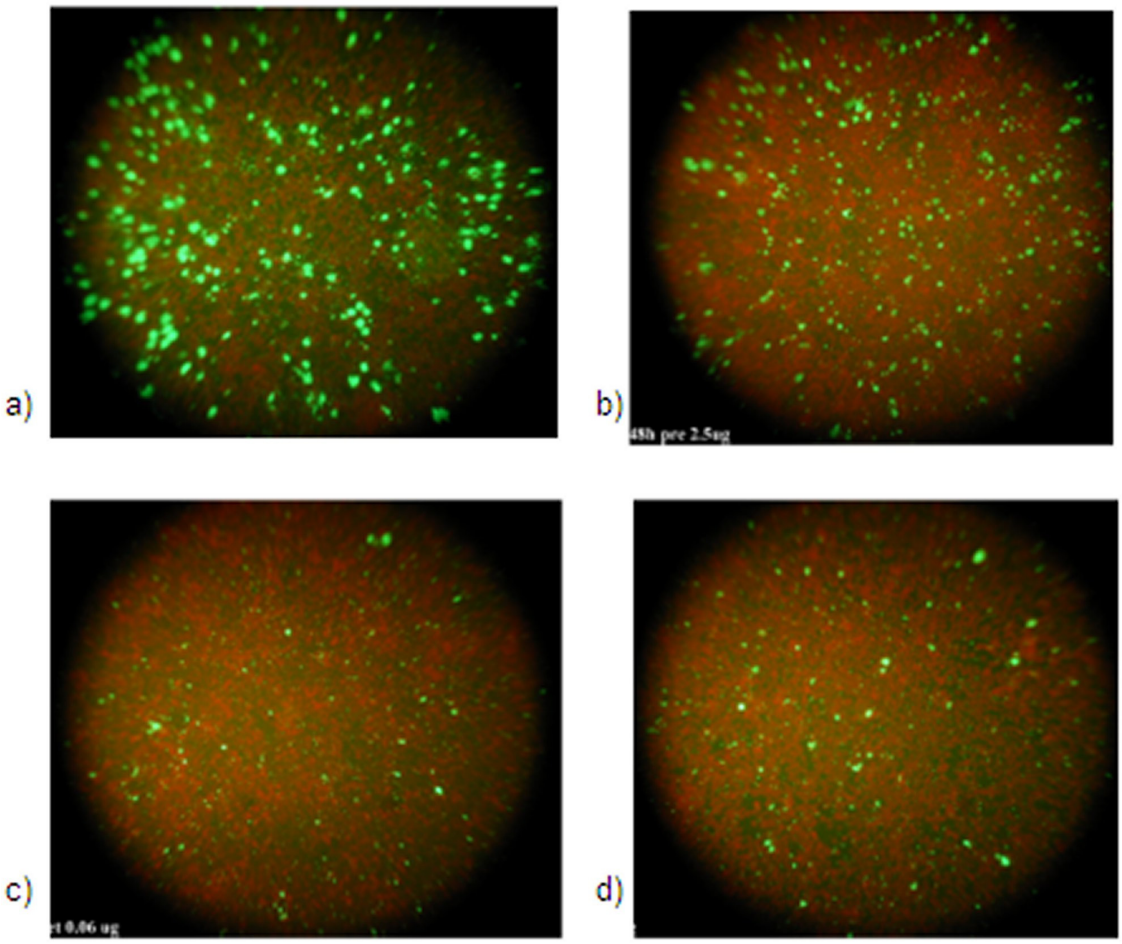


Figure 2-1. Reduction in fluorescent Chlamydial bodies *in vitro* after exposure to BDM-I.

Chlamydia 48 h after infection of McCoy cells (a) and when exposed to BDM-I at 2.5 µg/mL (b), 5 µg/mL (d), or 0.06 µg/mL tetracycline (c). Assay performed as described in Section 2.5.2.

alterations to 90% of inclusions (Stephens *et al.*, 1998). By this measure a reduction would have been seen at 0.06 µg/mL

BDM-I (5 µg/mL), did not inhibit reticulate body replication when administered 24 h post infection (results not shown). Tetracycline inhibited replication when administered at both time points. This suggests that BDM-I is interfering with binding and/or entry stages within the first few hours of infection but was not able to inhibit subsequent replication of chlamydial cells in inclusion membranes at the maximum concentration used in this assay. However, toxicity of BDM-I to McCoy cells precluded testing higher concentrations.

2.5.4 Discussion

Activity against this obligate bacterial pathogen in addition to the protozoal intracellular pathogens, *Plasmodium* and *Eimeria*, confirms that BDM-I acts intracellularly on a fundamental metabolic process common to prokaryotic and eukaryotic pathogens and/or their host cells.

The inhibition of early stage infection of McCoy cells but not reticulate body replication suggests that BDM-I is affecting one or more of the metabolic functions necessary for binding or endocytosis which could be an effect on a host cell mechanism coopted for entry or a target unique to *Chlamydia* spp. BDM-I was not shown to affect replication of EB's, which suggests that there is no direct interference with unique chlamydial or commandeered host cell metabolic functions involved in cell replication. However this is qualified by the observation that the level of BDM-I achievable in McCoy cells may be too low to inhibit these functions. Future work will be needed to replicate these results as replication was not possible in the given time-frame due to experimental complications. BDM-I, however, is effective against other intracellular Gram-negative pathogens such as *Neisseria* and *Haemophilus* at 1-5 μg/mL (Table 1-1), within the concentration level used in the *C. trachomatis* assay.

There is evidence that *Chlamydia* exploit host signalling and trafficking pathways for their development. Two open reading frames (ORF's) showing homology with serine threonine protein kinases and one ORF showing homology to a serine threonine protein phosphatase, PP-2C, have been identified (1997). No tyrosine phosphatases in *Chlamydia* have been identified. Fawaz et al. (2004) showed that chlamydial infection of epithelial cells with *C. trachomatis* resulted in tyrosine phosphorylation of several host proteins, including proteins involved in signal transduction and associated with the cytoskeleton, including cortactin. Active cortactin

is a tyrosine phosphorylated protein which is associated with the chlamydial vacuole early in pathogenic invasion. They showed that orthovanadate, a PTP inhibitor, inhibited infection of cells by *C. trachomatis*.

Tyrosine phosphorylation events occur very early in infection, consistent with a role in the entry step. Clifton *et al.* (Dautry-Varsat *et al.*, 2005) have identified a chlamydial actin-recruiting tyrosine phosphoprotein (TARP) which is expressed in EB's (unphosphorylated) and translocated into the host cell by the chlamydial TTSS. It is then rapidly phosphorylated and is associated with signalling events for actin recruitment and cytoskeletal reorganisation (Gupta & Griffiths, 2006; Stephens *et al.*, 1998).

Chlamydia possess a type III secretion system (TTSS), as do many intracellular pathogens. The TTSS is a protein export apparatus allowing proteins to be secreted across the cytoplasmic membrane. TTSS translocates microbial proteins into the cytosol of eukaryotic cells to facilitate bacterial pathogenesis. TTSS in *Chlamydia* is probably used to modify host cell processes involved in cell invasion, to modify the inclusion membrane and host cell regulatory pathways (Stephens *et al.*, 1998). Chlamydial phosphorylated proteins in the inclusion membrane, such as IncA may be phosphorylated by host kinases (Tuomanen *et al.*, 1986).

The significance of signalling events, particularly through phosphorylation by host kinases, of both host and chlamydial proteins, in binding, entry and formation of inclusion bodies suggests that the mechanism of BDM-I against *Chlamydia trachomatis* may be inhibition of host tyrosine phosphatases involved in entry of EB's into the host cell and formation of inclusion bodies. In Chapter 5 BDM-I is shown to inhibit tyrosine phosphatases in an enzymic assay. Since no ORF's for likely tyrosine phosphatases

have been identified in the chlamydial genome, any inhibition by BDM-I of PTP's is likely to be of host PTP's.

2.6 KILL RATES

2.6.1 Background

Rate of Kill

The Minimum Microbicidal Concentration (MMC) is defined as the lowest concentration producing a 3 $\log_{10}$ reduction in inoculum density within 24 h in the standard MIC assay. This also describes the microbicidal or microbistatic status of an agent, a microbicidal drug having an MMC ≤4× the MIC. This index does not provide information on kill kinetics at various concentrations at and above the MIC. Different rates of kill of susceptible microbial species can indicate differences in the metabolic significance of the target(s) of BDM-I. Rapid killing may indicate that the target is particularly important in survival of that species or that there is an additive effect between primary and secondary targets.

Time-kill assays for BDM-I were performed to investigate differences in the rate of kill of different microbial species and also provide a basis for treatment times and concentrations for later assays.

Effect of BDM-I on *S. aureus* at different population growth rates

In vitro assays for antimicrobial activity are usually performed in cells growing in optimal conditions. This measures the microbicidal ability on fast growing, rapidly metabolising cells which are more susceptible to inactivation of a pathway. The effects of a compound on slowly growing or dormant cells can be different. β-lactam

antibiotics, for example, have more pronounced effects on rapidly growing cells actively synthesising peptidoglycan (Craig, 1998).

Microbicidal drugs are characterised by fast killing of microbial cells *in vitro*. If this activity is time-dependent, concentrations above the MIC will make little difference to the rate of kill and a dose-response may not be evident (e.g. β-lactams, macrolides, oxazolidinones). This is in contrast to concentration-dependent killing where the bacterial load can be reduced faster by using increasing concentrations above the MIC (e.g. aminoglycosides, fluoroquinolones) (Eng *et al.*, 1991).

Time kill studies are usually determined for a fixed concentration of inhibitor against cells under optimal conditions and in the log or exponential phase of growth and therefore growing rapidly. However, this may not be indicative of microbicidal activity against organisms when conditions are not optimal and the growth rate is slow or cells are dormant, conditions characteristic for pathogens infecting hosts (Eng *et al.*, 1991).

Here, time-kill rates were determined in media supporting optimal, suboptimal growth rates or not supporting growth, to determine if BDM-I was more active against actively replicating cells and therefore affecting a metabolic activity essential to microbial replication or structural integrity. Often when the maintenance of cellular structural integrity or vital metabolism is targeted by an agent, the effects will be more pronounced in rapidly multiplying cells that have a greater metabolic demand.

2.6.2 Materials and Methods

Rate of Kill

Strains used were *B. cereus* RMIT 30/7, *B. subtilis* ATCC 6633, *S. aureus* ATCC 29213*, E. faecalis* ATCC 29212, *P. vulgaris* ATCC 13315*, M. catarrhalis* RMIT 211/2*, E. coli* ATCC 25922 and *C. albicans* ATCC 10231. Overnight cultures were

diluted 1/100 in MHB (SLM for *C. albicans*) and incubated at 37ºC on a platform shaker at 160 rpm (Ratek, Vic, AUS). Log-phase cultures were diluted in medium to approximately 1×10^6 CFU/mL. Sterile McCartney bottles were prepared with 5 mL medium with or without BDM-I at various multiples of the MIC_H and pre-warmed to 37ºC. Each was inoculated with 50 µL of the bacterial suspension. All test systems contained a final 1% v/v DMSO (BDH chemicals), including controls without BDM-I. All bottles were incubated at 37ºC, at 160 rpm on a platform shaker between sampling times. At time points 10 µL aliquots of 10-fold serial dilutions in saline were plated onto NA and plates incubated for 24 h at 37ºC aerobically to determine the number of viable cells per mL. Cultures were sampled at 0, 4, 8 and 24 h except for *M. catarrhalis* and *E. coli* (0, 3, 6, 24 h) and *E. faecalis* (0, 6, 8, 24 h). Each assay was completed twice and average viable counts reported.

Effect of BDM-I on *S. aureus* at different population growth rates

Based on the method of (Denisenko *et al.*, 27 Dec. 2002 International patent), time-kill curves were performed in medium formulations designed to support optimal, suboptimal and no population increase of *Staphylococcus aureus*, to identify the effect of a difference in growth rate on to BDM-I activity. MHB (Oxoid) was used as the control medium for comparison. Yeast Nitrogen Broth (YNB, Difco™, BD Biosciences), without a carbon source or amino acids, leaving a sole nitrogen source (5 g/L, ammonium sulfate), was used for the 'no population growth' state. The 'optimal growth' formulation consisted of YNB with 0.5% w/v glucose (Sigma-Aldrich) and 1.7% w/v Casamino acids (Difco™, BD Biosciences, CA, USA). The 'suboptimal growth' formulation consisted of YNB with 0.05% w/v glucose and 0.0017% w/v Casamino acids (Difco™, BD Biosciences).

Four McCartney bottles were each prepared with 5 mL of a formulation. BDM-I in DMSO was added at a concentration 100× that of the final test concentration (16 µg/mL) and the solution warmed to 37ºC overnight. The controls also contained a final concentration of 1% v/v DMSO. An overnight broth culture of *S. aureus* was diluted and each bottle inoculated with approximately 1×10^6 CFU/mL log phase bacteria. Ten-fold dilutions were plated onto NA and incubated at 37°C for 24 h to obtain viable counts. Cell numbers ($\log_{10}$ CFU/mL) were plotted over time and growth rates of cultures treated with BDM-I compared to rates of the control cultures.

2.6.3 Results

Rate of Kill

Time kill curves for BDM-I against selected bacteria and *C. albicans* are shown in Figure 2-2. The MIC_H was used as a reference value for reporting effective concentrations because this inoculum density was used for rate of kill studies. BDM-I reduced population growth in bacteria slowly and in a weakly dose-dependent manner in general. BDM-I concentrations above the lowest concentration, that were first to show bactericidal activity, usually resulted in little or no increase in the kill rate. There was no common pattern based on cell wall structure (Gram-reaction) for the bacterial species tested and there was considerable variability in killing rate for strains similarly susceptible when compared by MIC values.

BDM-I was most rapidly bactericidal against *B. subtilis* and *B. cereus*, with concentrations 4× MIC_H producing a 5.5 $\log_{10}$ and 3 $\log_{10}$ reduction in viable cells over 24 h respectively. A less rapid kill over 24 h for concentrations ≥2× MIC_H was observed for *M. catarrhalis* and *P. vulgaris*, BDM-I reducing cell numbers by 3-4 $\log_{10}$ and showing no dose response. BDM-I was much less bactericidal to the Gram-positive

cocci, failing to reduce cell numbers of staphylococci at 16× the MIC_H and reducing cell number of enterococci by 1 $\log_{10}$ at 8× MIC_H.

The yeast, *C. albicans* was killed more rapidly than the bacterial strains tested with a 5 $\log_{10}$ reduction in 8 h at 2× the MIC_H. Only *C. albicans* showed a clear increase in kill rate with concentration. The fungicidal action on *C. albicans* confirms the low minimal fungicidal concentrations for fungi which were generally the same as, or 2-fold higher than the MIC (Table 1-1).

Effect of BDM-I on *S. aureus* at different population growth rates

The growth rates of *S. aureus* in the broth formulations permitting optimal and suboptimal growth can be seen in Figure 2-3a. There was a 1 $\log_{10}$ reduction in cell numbers in suboptimal growth conditions between 6 h and 24 h. BDM-I at 16 µg/mL (2× MIC_H) to YNB (which has no carbon source) did not kill non-replicating cells (Figure 2-3b). BDM-I, at 16 µg/mL, was equally bactericidal under both suboptimal and optimal growth conditions, producing a similar 1 $\log_{10}$ reduction in cell numbers between 6 h and 24 h. This suggests BDM-I only kills metabolising cells but is attacking a target not immediately essential to cell replication such as cell wall, protein and DNA synthesis. The lack of kill of dormant cells confirms that BDM-I is not directly degrading cell wall or membranes. It is also consistent with the lack of a dose response in the kill rate for bacteria.

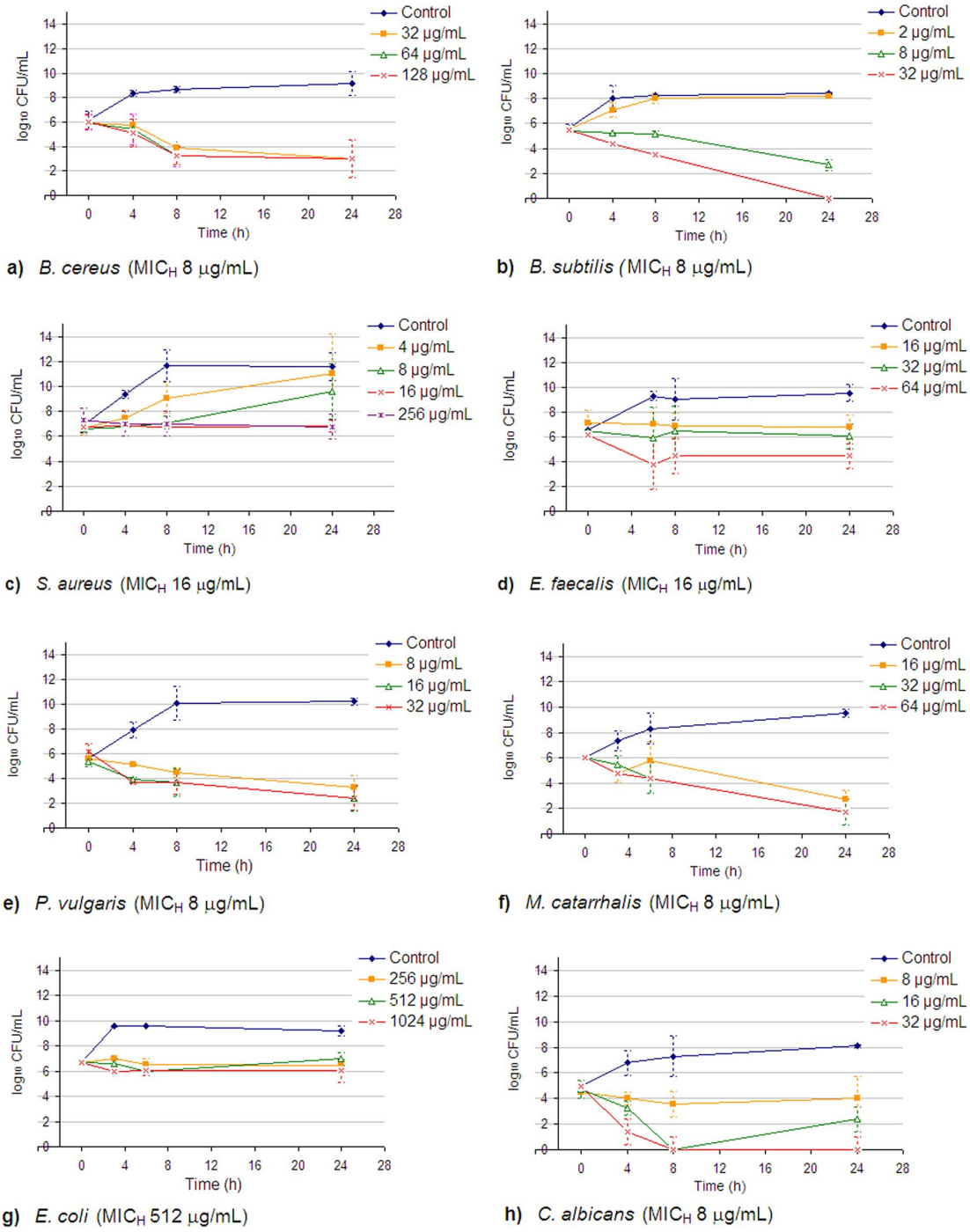
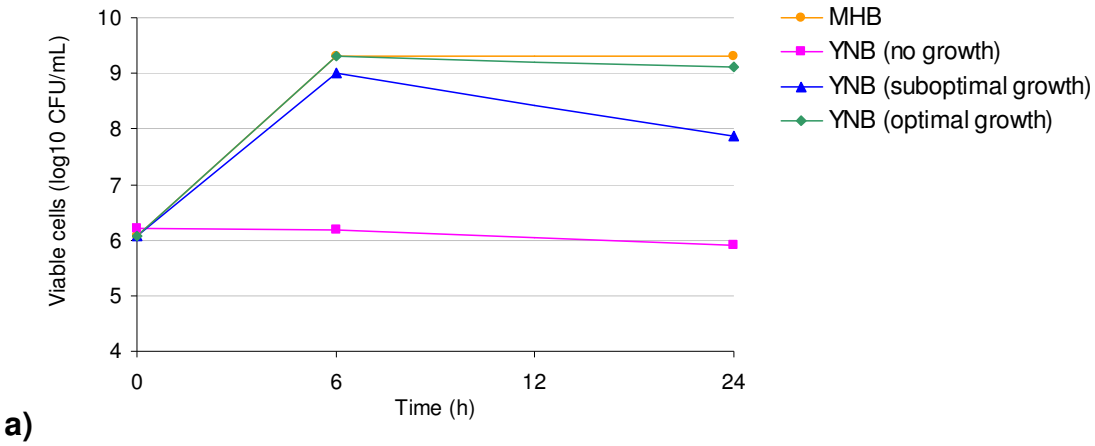
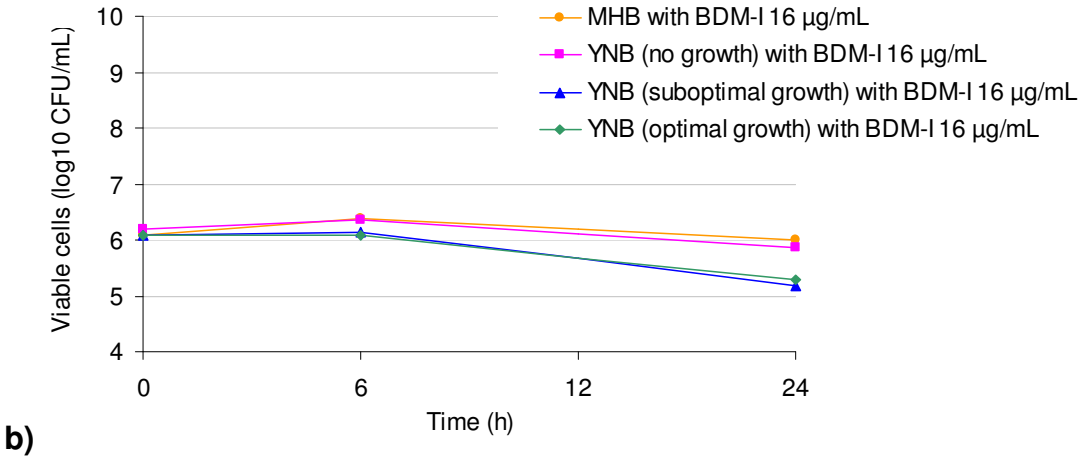


Figure 2-2. Time-kill curves for BDM-I against bacteria and *Candida albicans*.

Bacterial time-kills were performed in MHB and *C. albicans* in SLM as described in Section 2.6.2. Each line represents BDM-I concentrations in µg/mL. Controls contained media (1% v/v DMSO) without BDM-I. Error bars represent standard deviation.



a)



b)

Figure 2-3. Effect of BDM-I on *S. aureus* at different population growth rates.

The rate of growth in MHB in broth formulations without BDM-I is shown in a). The effects of 16 µg/mL BDM-I on *S. aureus* in MHB and in media supporting no growth, suboptimal growth and optimal growth are shown in b). Broth formulations were as follows: no growth, YNB without additives; suboptimal growth, YNB with 0.01% w/v glucose and 0.0017% w/v Casmino acids; optimal growth, YNB with 0.5% w/v glucose and 1.7% w/v Casmino acids. Broth formulations and assay method are described in Section 2.6.2.

2.6.4 Discussion

Rate of Kill

BDM-I is not uniformly bactericidal to susceptible species, the MMC being much more variable across genera than the MIC. For example, BDM-I is bacteriostatic against *Staphylococcus* and *Enterococcus* spp. and bactericidal to *Streptococcus* spp. with a similar or lower MIC (Denisenko *et al.*, 27 Dec. 2002 International patent). Bactericidal activity in the Gram-negative bacteria is variable, BDM-I being bactericidal to the fastidious species but not to several enteric and related species. This differentiates BDM-I from broad spectrum biocides which kill cells directly and uniformly.

The bactericidal action of BDM-I is minimal or slow and is little affected by increases in the concentration. This suggests a target or targets not immediately significant to cell viability or cell replication, but that over time is/are important to cell survival. The differential in kill rates between species suggest that the target varies in significance for cell survival between species or that different or additional targets are attacked across species.

Since the site of action of BDM-I is intracellular and not on the cell wall or cell membrane of bacteria, as established from other studies (Chapters 2 and 3), entry rate into the cell and accessibility to the target could be a factor in the slow kill rate. However, variation in kill rate between species with a similar wall structure (Gram-positive) or envelope structure (Gram-negative) and all with very similar cytoplasmic membranes, suggests compound entry rate into the cell is not a factor.

C. albicans, in contrast, was rapidly killed within 4 hours and showed a clear dose response (Figure 2-2). This suggests the targets are more directly related to cell replication in eukaryotic microorganisms than in prokaryotes. BDM-I is more

uniformly active across fungal species (Table 1-1) and is fungicidal to all species tested at concentrations at or 2-fold above the MIC (Eng *et al.*, 1991). The difference in microbicidal activity between fungi and bacteria could be explained by a difference in target or in the significance of the target to growth and replication, between eukaryotic and prokaryotic cells. The broad antimicrobial spectrum of BDM-I favours a common major MOA. A proposal that this major target is cellular protein tyrosine phosphatases is made in Chapter 5, where the occurrence and significance of PTP's in eukaryotic and prokaryotic cells is discussed. Protein tyrosine phosphatases have been shown to be involved in cell growth, cell differentiation and the cell cycle in eukaryotic cells. The important role of PTP's in eukaryotic cell growth could explain the more rapid microbicidal activity observed in *C. albicans.*

Effect of BDM-I on *S. aureus* at different poopulation growth rates

In common with many bactericidal antibiotics, BDM-I does not kill dormant cells. This differentiates it from broad spectrum biocides which kill microbial cells directly. The β-lactams, fluoroquinolones and aminoglycosides exert a maximum effect on rapidly growing cells and kill rates are considerably reduced with suboptimal growth conditions (Pankey & Sabath, 2004). BDM-I, although slowly bactericidal, has the advantage of being equally effective in killing slowly replicating cells, an advantage for an anti-infective drug because growth rates are usually initially slow and infected tissues contain a dense population of non-growing bacteria (Denyer & Hugo, 1991). Failure to kill infecting cells can lead to latent or chronic infections if cells are inaccessible to host defence mechanisms. Slow bactericidal activity is not always a disadvantage in an anti-infective drug. Other than in severe and sudden infections,

adequate and continuing control of replication can be sufficient to tip the advantage in favour of host defence mechanisms.

2.7 INTERACTIONS WITH ANTIBIOTICS

2.7.1 Background

Interactions between antibiotics can be investigated for antagonistic or synergistic effects on target bacteria. The interaction between two compounds may indicate a relationship between their mechanisms of action (Odds, 2003). In combination, drugs with synergistic effects are required in less concentrated amounts than either drug alone. Synergism can arise when the mechanisms of the two compounds are directly complementary (affecting the same metabolic function) or indirectly complementary (affecting a necessary related function). Antagonistic reactions are observed where the addition of one drug increases the concentration required of the second drug. This may be due to interference of the second drug's mechanism by the mechanism of the first. For example, most antibiotics are bacteriostatic against enterococci, however a synergistic effect between a cell-wall active β-lactam agent and an aminoglycoside, targeting the ribosome can produce a bactericidal effect through increased penetration of the aminoglycoside.

The checkerboard titration is an efficient technique for demonstrating synergism (greater effect in combination) or antagonism (lesser effect in combination) between two compounds (Sweeney & Zurenko, 2003). Interactions between BDM-I and representatives from the major classes of antibiotics were investigated for antagonistic or synergistic effects on selected bacteria by chequerboard titration and calculation of Fractional Inhibitory Concentration Indices (FICI). Interactions were

further investigated by determining time-kill kinetics for drug combinations shown to interact in checkerboard titrations.

2.7.2 Materials and Methods

Synergism or Antagonism of BDM-I with antibiotics

BDM-I was investigated for interaction with a protein synthesis translocation inhibitor (erythromycin), an aminoacyl-tRNA inhibitor (tetracycline), a glycopeptide inhibitor of cell wall synthesis (vancomycin) and a quinolone (ciprofloxacin) that interferes with DNA gyrase supercoiling.

Strains used were *S. aureus* ATCC 29213*, E. faecalis* ATCC 29212 and *E. coli* ATCC 25922. Each test strain was grown to log phase in MHB at 37°C and diluted to a final inoculum density of $1 \times 10^5 - 1 \times 10^6$ CFU/mL in a total of 200 µL. Antibiotics and BDM-I were prepared as 2-fold dilutions at 4× test concentrations in MHB. Microtitre plates (Sarstedt Australia) were prepared with 50 µL of increasing concentrations of the two compounds (i.e. BDM-I and one antibiotic) on adjacent axes. Inoculated plates were incubated at 37°C for 20 h and MIC's for each compound alone and in combination were recorded. The pattern of inhibition was indicative of the interaction between the two compounds and the method enables the calculation of a Fractional Inhibitory Concentration Index (FICI), a numerative interpretation of the type of interaction displayed.

For wells containing the lowest inhibitory combinations of drugs, a Fractional Inhibitory Concentration (FIC) is derived for each well from the calculation:

$$\frac{\text{MIC of compound A with compound B}}{\text{The MIC of compound A alone}} + \frac{\text{MIC of compound B with compound A}}{\text{The MIC of compound B alone}}$$

The mean of all FIC values for each plate gives the FICI, a numerative interpretation of synergistic, antagonistic or indifferent interaction. An FIC Index of ≤0.5 was considered to indicate synergism, a value ≥4 to indicate antagonism and all values >0.05 to <4.0 indicated an indifferent interaction (Kadota, 1996; Pasquale & Tan, 2005; Williams, 2001). All assays were performed on at least three different occassions.

Time-kill interaction between an antibiotic and BDM-I

Any interaction observed between an antibiotic and BDM-I against any strain was further investigated using a time-kill method in MHB at 37ºC as described in Section 2.6.2. BDM-I was added from 100× working stock solutions in DMSO and equal concentration (µg/mL) of the antibiotic added. Ratios of BDM-I and drug were mixed at 1:1 (µg/mL) with concentrations equal to ½, 1×, 2×, 4×, 8× and 16× the MIC_H. All tubes contained a final DMSO concentration of 1% v/v including the MHB control without BDM-I. All tubes were incubated for at least 15 min at 37°C before addition of inoculum. The test strain was grown overnight in MHB at 37ºC. The overnight culture was then diluted in MHB, incubated to early log phase and used to inoculate 5 mL MHB to a density of approximately 5×10^6 CFU/mL. All bottles were incubated at 37ºC, at 160 rpm on a platform shaker (Ratek) between sampling times. At 0, 8 and 24 h, 10 µL aliquots of 10-fold serial dilutions in saline were plated onto NA and plates incubated for 24 h at 37ºC aerobically to determine the number of viable cells per mL. The assay was completed twice and average viable counts reported.

2.7.3 Results

Synergism or Antagonism of BDM-I with antibiotics

Checkerboard titration assays did not show synergistic or antagonistic interactions between BDM-I and erythromycin, ciprofloxacin or vancomycin. Borderline synergism was observed for concentrations of BDM-I and tetracycline with a mean FICI of 0.51. This interaction was at the weaker end of the spectrum, being close to the maximum FICI value for a synergistic reaction 0.5. It was further investigated by assessing the effect of combinations of tetracycline on the rate of kill of *E. faecalis*.

Table 2-3. Average FIC Indices resulting from chequerboard titrations between BDM-I and selected antibiotics.

Antibiotic:	Tetracycline		Erythromycin		Ciprofloxacin		Vancomycin	
Bacteria	Av. FICI (±SD)	Interpretation	Av. FICI (±SD)	Interpretation	Av. FICI (±SD)	Interpretation	Av. FICI (±SD)	Interpretation
E. faecalis	0.51 ±0.12	Weak Synergism /Indifferent	0.75 ±0.22	Indifferent	1.09 ±0.16	Indifferent	1.03 ±0.29	Indifferent
S. aureus	0.92 ±0.25	Indifferent	1.4 ±0.4	Indifferent	0.99 ±0.25	Indifferent	1.15 ±0.3	Indifferent
E. coli	2.19 ±0.62	Indifferent	—	—	2.46 ±0.24	Indifferent	—	—

FIC Index Interpretations: ≤0.5, synergism; >4, antagonism; >0.5 to <4.0 indifferent interaction.
SD, standard deviation.

Time-kill interaction between an antibiotic and BDM-I

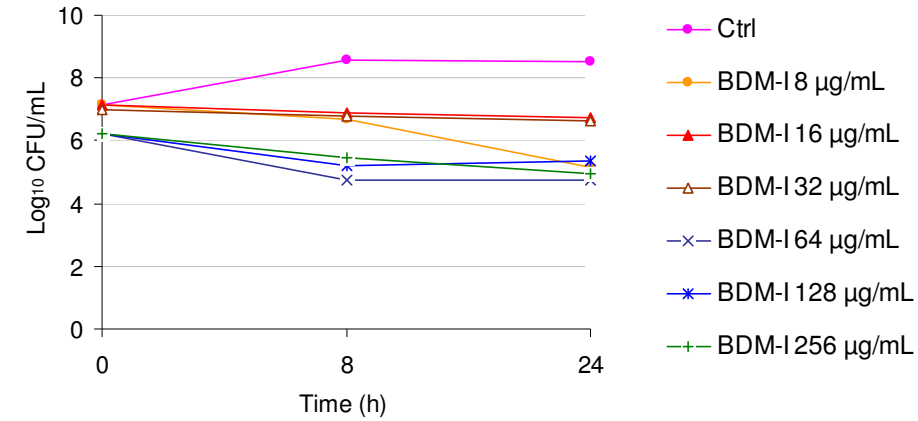
The time-kill study on the interaction between BDM-I and tetracycline against *E. faecalis* showed antagonistic rather than the synergistic activity observed in the checkerboard titration). BDM-I produced a reduction (~1 $\log_{10}$) in cell numbers at concentrations from 64 to 256 μg/mL, showing very little increase in kill rate at increasing concentrations from 4× to 32× above the MIC (16 μg/mL) which confirms

the observation in the time kill studies above (Section 2.6.3). Tetracycline was much more rapidly bactericidal and showed a dose response from 16 to 256 µg/mL, with a 4 $\log_{10}$ reduction in viable count at a concentration of 64 µg/mL, 4× the MIC (16 µg/mL) determined during checkerboard titrations. At equal concentrations, the rate of inhibition was comparable for both compounds alone (Figure 2-4a & b) and together in combination (Figure 2-4c). At equal concentrations (>32 µg/mL each), BDM-I had a small antagonistic effect on tetracycline resulting in lower kill rate than for tetracycline alone. BDM-I at 2× MIC was also tested in the presence of tetracycline at 0.6×, 2.6× and 10× MIC and showed a similar antagonism (results not shown).

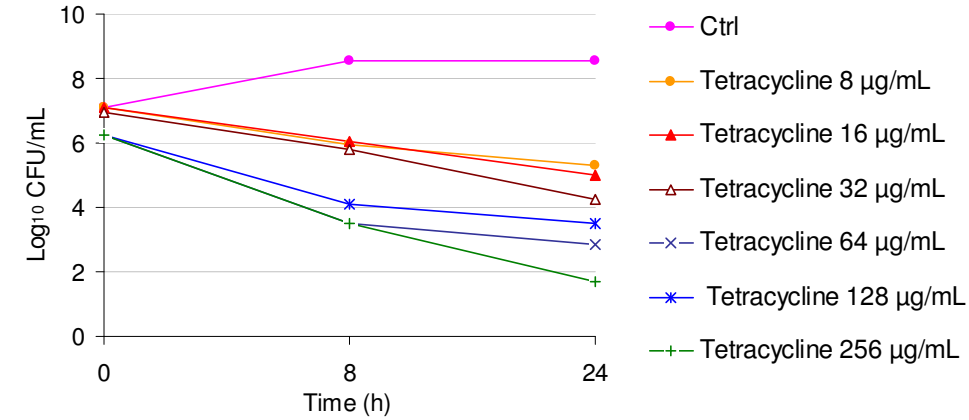
2.7.4 Discussion

The lack of any significant interaction of combinations of BDM-I with traditional antibiotics suggest that BDM-I is not affecting metabolic targets relating to the pathways targeted by these agents. This supports a novel MOA for BDM-I. The suggestion of possible weak synergism of tetracycline with BDM-I from the chequerboard titration is currently not explained. BDM-I could be acting on a metabolic function which interferes with a target of tetracycline other than inhibition of ribosomal function.

Antibiotics have many non-antimicrobial effects on eukaryotic cells and side effects in humans (Roberts, 2003). Tetracycline is unusual among antibiotics in having a very broad antibacterial spectrum including the intracellular chlamydiae, mycoplasmae and rickettsiae and being active against some intracellular protozoal parasites while also being used to treat some non-infectious diseases (Madigan *et al.*, 2006). This suggests that there may be unidentified effects in bacteria these two antibiotics which bind transiently that may explain the interactions reported here.



a)



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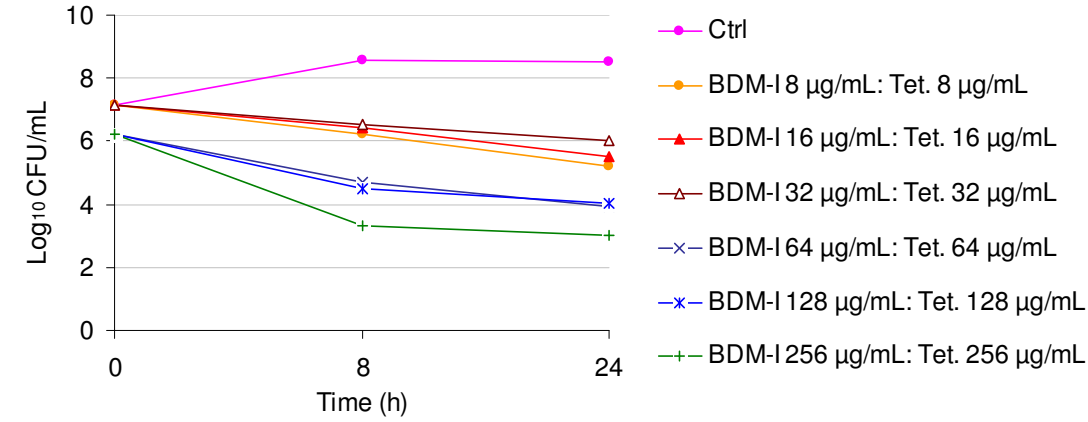


c)

Figure 2-4. Effect of BDM-I and tetracycline in combination on the rate of kill of *Enterococcus faecalis.*

a) Effect of BDM-I (µg/mL) alone on *E. faecalis*; b) Effect of tetracycline (µg/mL) alone on *E. faecalis*; c) Effect of BDM-I & tetracycline (µg/mL) in equal proportions on *E. faecalis*.

CHAPTER 3 THE EFFECT OF BDM-I ON CELLULAR MEMBRANES

3.1 INTRODUCTION

Bacterial membranes

Cell wall and membrane structures are a major defence against harmful agents. The differences between prokaryotic and eukaryotic cell structure have made wall and membrane components attractive targets for selective agents.

Gram-positive bacteria possess a cell wall composed of 90% peptidoglycan (Madigan *et al.*, 2006). This thick layer, external to the cytoplasmic membrane, provides a hydrophilic outer surface capable of resisting harsh environments whilst being porous to most molecules. External to the cell wall there may also be a capsule or slime layer composed of high molecular weight polysaccharides or polypeptides, which are important virulence factors. In Gram-negative bacteria there is a much thinner peptidoglycan layer (10 %) surrounded by a lipid bilayer constituting an outer membrane (or envelope) and enclosing the periplasmic space (Nakae & Nakae, 1982; Thanassi *et al.*, 1995; Toro *et al.*, 1990). The OM is impermeable to large molecules, selectively permeable to hydrophilic molecules and highly selective and slowly permeable to lipophilic molecules. The outer leaflet of the OM is composed of lipopolysaccharides and proteins, while the inner leaflet is a complex of lipoproteins and phospholipids, similar to the cytoplasmic membrane. The outer leaflet lacks phospholipids that allow diffusion of lipophilic molecules. It contains porins for passage of low molecular weight hydrophilic molecules such as sugars and amino acids. They may also allow passage of some small antibiotics, such as tetracyclines,

aminoglycosides and chloramphenicol (Nikaido, 1989). Bacterial species vary with respect to the permeability due to porins, that of *Pseudomonas spp.* being very low (Madigan *et al.*, 2006). The structure and composition of the outer wall differ widely between species and are of major significance in susceptibility to antimicrobial agents.

The cytoplasmic membrane is a semi-permeable phospholipid bilayer, embedded with integral proteins, which has complex biological functions. It is responsible for transport of metabolites and solutes into and out of the cell and contains membrane-bound proteins and enzymes performing many functions in bacteria which are carried out in the cytoplasmic organelles of eukaryotic cells (Madigan *et al.*, 2006). The cytoplasmic membrane is highly conserved across microbial species and is likely to be uniformly susceptible to membrane active agents which have access to it. Access to the membrane will however vary depending on the chemical nature of the agent and the cell wall or OM structure of microorganism as described above.

Fungi have cell walls that are structurally similar to plant cell walls though their composition differs. They are typically ≥80% polysaccharide, with the main constituent being chitin and also contain proteins, lipids, polyphosphates and inorganic ions (Madigan *et al.*, 2006). The cytoplasmic membrane in fungi differs from that of bacteria in that ergosterol, rather than substances such as hopanoids in bacteria, regulate membrane fluidity and stability (Maillard, 2002).

Agents disrupting the cell wall or membrane

Damage to the cell wall and cytoplasmic membrane is a key action of some therapeutic agents and many disinfectants. Microbicidal activity by membrane-active agents may involve physical disruption, dissipation of the proton motive force or inhibition of membrane-associated enzymes (Maillard, 2002). Disinfectants, such as phenolics, biguanides, quaternary ammonium compounds, alcohols and cationic

surfactants act primarily on the cytoplasmic membrane causing leakage and/or coagulation of the cytoplasm. At low concentrations, well below their concentration of use, many disinfectants have been shown to have more specific actions, such as interference with membrane enzymes and biosynthetic capabilities (Odds *et al.*, 2003).

Anti-infective agents are much more selective in their action on membranes than biocides. Gram-positive bacteria are susceptible to penicillins, cephalosporins and glycopeptides which inhibit the synthesis of cell wall peptidoglycan. Wall disruption results in secondary damage to the cell wall and ultimately cell lysis. Polymyxin B, a cationic cyclic lipopeptide, binds to the lipid A portion of lipopolysaccharide in the Gram-negative envelope, disrupting and damaging the OM. Daptomycin inserts into the cytoplasmic membrane, disrupting membrane potential, resulting in cell leakage and multiple downstream effects. Anti-fungal agents such as the imidazoles (ketoconazole), the triazoles (fluconazole) and the polyenes (nystatin and amphotericin B) inhibit biosynthesis of ergosterol weakening membrane stability (Oliva *et al.*, 2003).

3.2 CYTOPLASMIC MEMBRANE INTEGRITY

3.2.1 Background

Whole cell lysis

Cell lysis can be a direct effect of degradation of the cytoplasmic membrane or a secondary autolysis resulting from disruption of the protective wall or envelope, for example by agents interfering with synthesis of peptidoglycan. Depolarisation of the membrane leading to autolysis by daptomycin has been shown to be a new mechanism of membrane damage. Lysis by wall-active agents may be slow while direct degradation of the membrane results in rapid lysis.

The effect of BDM-I on the integrity of the cytoplasmic membrane of *B. cereus* and *C. albicans* was assessed using a whole cell lysis assay. The species were selected because they are rapidly killed by BDM-I (section 2.3.3). The inclusion of *C. albicans* enables comparison of activity on both bacterial and fungal cells. In suspensions of bacterial and yeast cells, turbidity correlates directly with whole cell density and measures both living and dead cells. Lytic activity can be shown by a decrease in viable cells accompanied by a decrease in turbidity and is most easily demonstrated at high concentrations where there is little or no increase in turbidity.

Lysis of spheroplasts

Direct lysis of the cytoplasmic membrane can be investigated using bacterial spheroplasts. Spheroplasts are Gram-negative bacteria in which nearly all of the OM has been removed. Lysozyme destroys peptide bonds in peptidoglycan and weakens the wall. Protoplasts of Gram-positive bacteria can then be produced by adding sucrose to balance solute concentrations inside and outside the cell allowing lysozyme to digest peptidoglycan without cell lysis. In treating Gram-negative bacteria, EDTA (Ethylenediaminetetraacetic acid) is needed to disrupt the OM and allow access of lysozyme to the peptidoglycan layer. Spheroplasts have increased sensitivity to membrane damage, making them a useful indicator of agents directly active against the cytoplasmic membrane. A decrease in the optical density of a protoplast or spheroplast suspension in the presence of a compound allows direct measurement of cell lysis due to disruption of the cytoplasmic membrane (1999).

The ability of BDM-I to lyse spheroplasts of *E. coli* and *M. catarrhalis* was investigated. *E. coli* has low susceptibility to BDM-I and *M. catarrhalis* is very susceptible. They have differing levels and types of LPS in the outer envelope which could affect access of an agent to the cell membrane.

3.2.2 Materials and Methods

Whole cell lysis assay

C. albicans and *B. cereus* colonies were inoculated into 5 mL of SLM and MHB respectively and incubated at 37°C aerobically for 2 h with agitation. A 96-well flat bottomed microtitre plate (Greiner Bio-One) was prepared with BDM-I in media at 4× the test concentrations of 32, 64 and 128 µg/mL in 4% v/v DMSO. Each culture (100 µL) was added to microtitre wells and the plate incubated aerobically at 37°C for up to 8 h. Blank wells received no bacteria. Optical density at 600 nm (OD_{600nm}) was read at room temperature at the time of inoculation, 15, 30, 45 min and 1, 2, 4, 8 h after inoculation on a spectromphotometer (Cary 50 UV-Vis Spectrophotometer, Varian, Vic, AUS). The plate was kept at 37°C between readings. Microbicidal activity was measured by plating dilutions of the wells (10-fold) onto NA at 0, 2, 4 and 8 h and incubating plates aerobically at 37°C for 24 h for viable counts. Microbicidal lysis is indicated by a loss of turbidity and a fall in viable count. Decrease in viability with no loss in turbidity of the culture indicates a non-lytic attack on cells.

Lysis of Gram-negative spheroplasts

E. coli (MIC 512 µg/mL) and *M. catarrhalis* (MIC 2 µg/mL) were grown in 10 mL of MHB, incubated aerobically overnight at 37°C on a shaker at 160 rpm (Ratek). Each culture (100 µL) was used to inoculate 20 mL fresh medium and incubated at 37°C on a shaker (Ratek) at 160 rpm for 2 h. Log cultures were centrifuged at 2000 ×g for 5 min at 4°C (3K15, Sigma Laborzentrifugen) and washed twice in cold 50 mM Tris, pH 8 ($C_4H_{11}NO_3$, Amresco, Solon, Ohio, USA) with 20% w/v sucrose (AnalaR®, BDH Chemicals). Washed cells were resuspended in 5 mL of 50 mM Tris, pH 8, with 20% w/v sucrose and each divided into 2× 2.5 mL for

preparation of whole cells or spheroplasts. Spheroplasts were prepared according to the method of Ganzle *et al*. (Helander & Mattila-Sandholm, 2000) by incubation with 0.6 mg/mL lysozyme (500 µL, 5 mg/mL in 50 mM Tris, pH 8; Sigma-Aldrich) with 20% w/v sucrose and 0.06 M EDTA (1 mL, 0.25 M, Amresco, Solon, Ohio, USA) for 30 minutes at 37°C. Cells were checked for spherical appearance indicating absence of cell envelope with a phase contrast microscope. Spheroplasts were collected by centrifugation at 500 ×g for 5 min and resuspended in 3 mL 50 mM Tris (pH 8) with 20% w/v sucrose and 10 mm $CaCl_2$ (BDH Chemicals). Whole cell suspensions received 1.5 mL 50 mM Tris (pH 8) with 20% w/v sucrose (instead of lysozyme and EDTA) solutions and were incubated for 30 minutes at 37°C.

Resuspended spheroplasts and whole cells were adjusted to an OD_{570nm} of 0.2 and 100 µL of each added to a clear, flat-bottomed microtitre plate (Greiner Bio-One) in duplicate wells. BDM-I, 10 µL in 50% DMSO/PBS (Appendix A) was added to test wells for both preparations at 10× the desired final concentration. To the control, 10 µL of 50% DMSO in PBS (pH 7.4) was added in place of BDM-I. The final concentration of DMSO in all wells was 5% v/v. Turbidity was monitored spectrophotometrically at 570 nm every 10 minutes for 1 h (Cary 50 UV-Vis Spectrophotometer, Varian).

The percentage of intact spheroplasts or whole cells was calculated as:

(sample OD at time X / sample OD at time 0)*100

The experiment was repeated four times and calculations made based on average percentages of intact spheroplasts or whole cells. Decrease in the OD of the suspension after addition of a membrane-active agent indicates lysis of spheroplasts.

3.2.3 Results

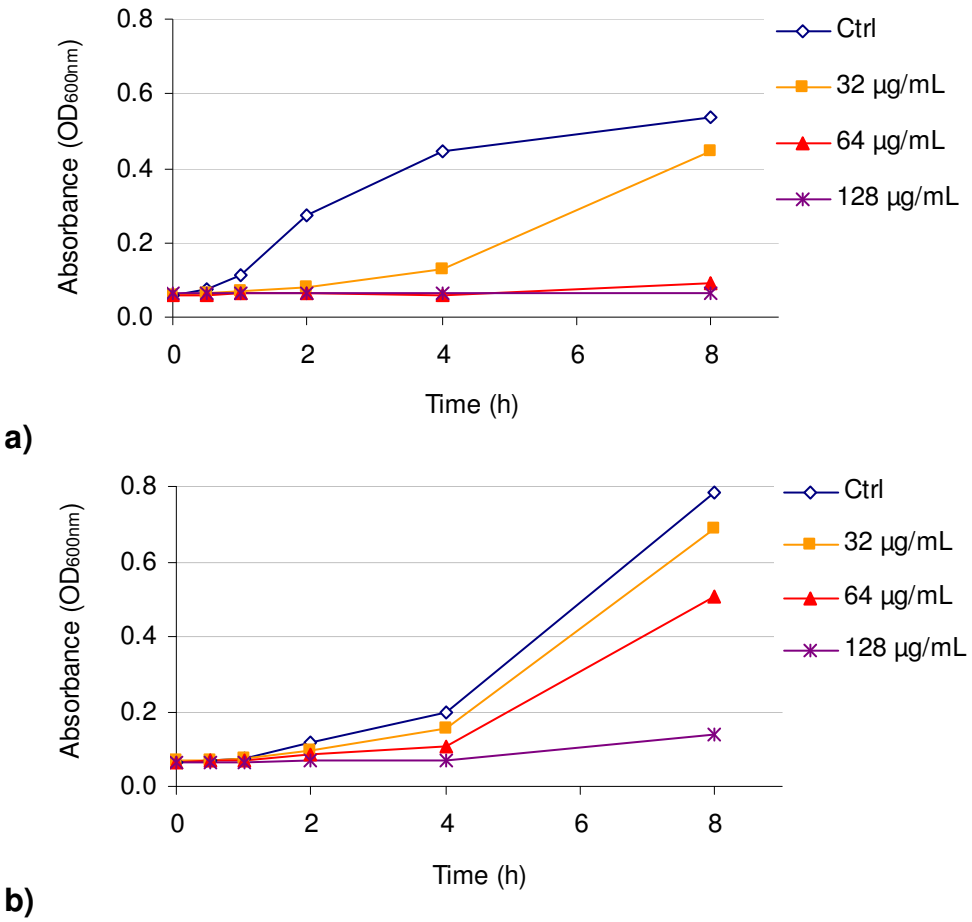
Whole cell lysis assay

Lytic activity can be shown by a decrease in viable cells accompanied by a decrease in turbidity (usually rapid) and is most easily demonstrated at high concentrations where there is little or no increase in turbidity. BDM-I at 128 µg/mL (16× the MIC_H) reduced the viable count of both *B. cereus* and *C. albicans* without loss of turbidity, indicating non-lytic microbicidal activity (Figure 3-1). The OD of cell suspensions exposed to BDM-I (8 - 128 µg/mL) gradually increased over 8 h in a dose-dependent manner. For both species tested, at 4× and 8× the MIC for BDM-I, OD_{600nm} continued to increase despite no increases in viability. During the assay, at 8 h, concentrations of 16× the MIC_H for BDM-I resulted in a decrease in viable cells of *B. cereus* and *C. albicans* of 3 $\log_{10}$ and 4 $\log_{10}$ respectively (data not shown).

Lysis of Gram-negative spheroplasts

Lysis of spheroplasts shows a direct action on the cell membrane which is not secondary to breakdown of the cell wall. Lysis of spheroplasts compared to lysis of whole cells indicates whether the OM is a barrier to penetration of an agent.

E. coli formed ≥80% spheroplasts after treatment with lysozyme and *M. catarrhalis* (~70%). There was no difference in the OD of suspensions of intact spheroplasts compared to whole cells of *E. coli* or *M. catarrhalis* in the presence of BDM-I (Figure 3-2). BDM-I caused no disruption of the cytoplasmic membrane of Gram-negative spheroplasts, confirming the observed lack of lytic action for whole cells of a Gram-positive bacterium and a yeast.



a)

b)

Figure 3-1. Optical density of *Bacillus cereus* and *Candida albicans* cultures continuously exposed to BDM-I.

B. cereus (a) was assayed in MHB, *C. albicans* (b) in SLM. BDM-I concentrations (µg/mL) in the media are shown as individual lines on the graphs. OD_{600nm} measurements carried out as described in Section 3.2.2. MIC_H for *B. cereus* 8 µg/mL, MIC_H for *C. albicans* 8 µg/mL.

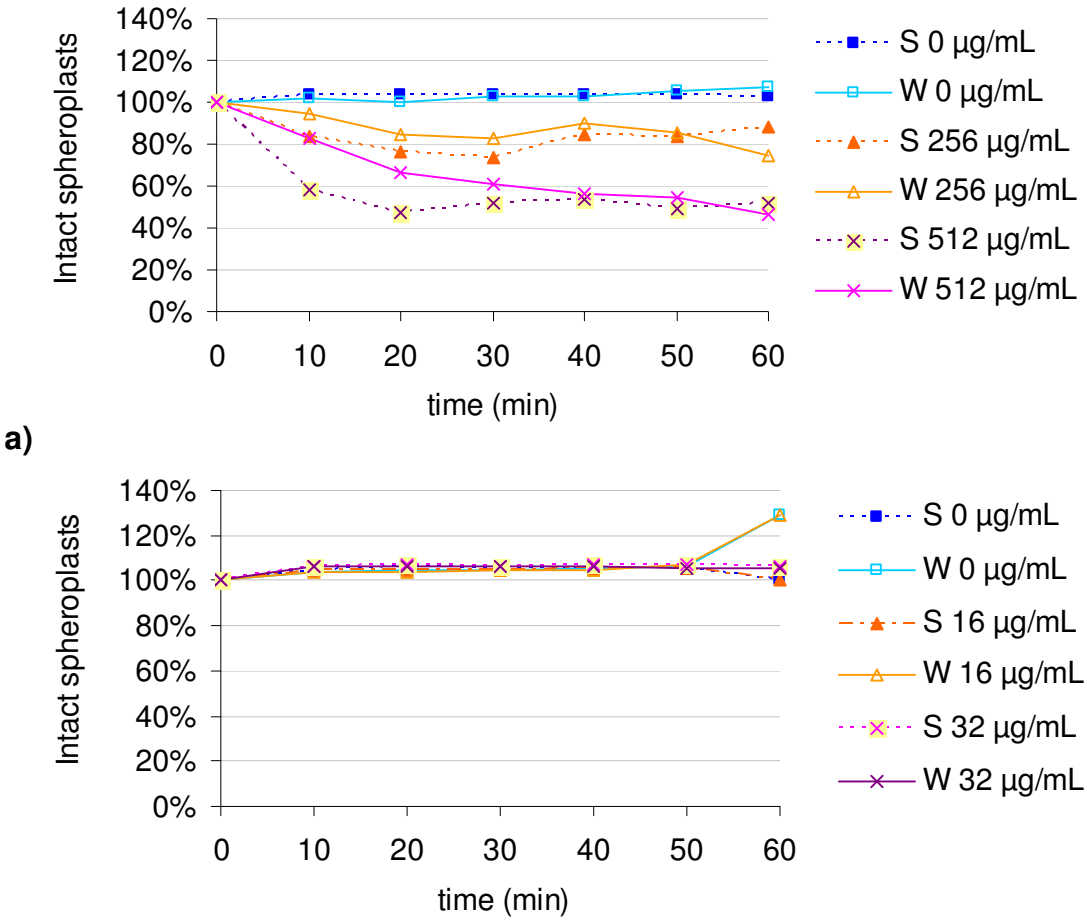


Figure 3-2. Percentage of *Escherichia coli* and *Moraxella catarrhalis* spheroplasts and whole cells remaining intact during exposure to BDM-I.

E. coli (a) and *M. catarrhalis* (b) spheroplasts (S) and whole cells (W) remaining intact in the presence of BDM-I (µg/mL). Assays were performed in Tris buffer with 20% w/v sucrose by OD_{570nm} as described in Section 3.2.2.

3.2.4 Discussion

Whole cell and spheroplast lysis

BDM-I is a small, highly lipophilic, neutral molecule which should readily diffuse through the lipid bilayers of microbial cells. It appears to do so readily and without disrupting the cytoplasmic membrane of *E. coli*. Cell lysis can be due to disruption of the cytoplasmic membrane or secondary to disruption of the cell wall. Spheroplasts, lacking the protection of the cell OM, are highly sensitive to disruption and degradation. The plasma membranes of bacteria and yeast are very similar and the results for *E. coli* spheroplasts are highly likely to apply to bacterial and yeast protoplasts. BDM-I does not lyse or crenellate red blood cells or affect the membranes of mammalian cells at concentrations which kill invading parasites (Nicoletti *et al.*, unpublished). This supports its lack of effect on bacterial spheroplasts. These assays suggest that BDM-I does not degrade the cell wall or cytoplasmic membrane of *B. cereus* and *C. albicans,* either directly or as a secondary effect of its microbicidal activity.

3.3 PERMEATION OF THE GRAM-NEGATIVE CELL ENVELOPE BY NPN

3.3.1 Background

The OM of Gram-negative bacteria is a significant permeability barrier protecting the cytoplasmic membrane. Its composition and significance to resistance to lipophilic agents is discussed above and in Section 2.3.1. Several anti-infective agents are able to permeate the OM and can be used to treat Gram-negative bacterial infections. BDM-I has shown variable activity against Gram-negative pathogens which

may partly reflect a barrier effect by the OM. Several substances, such as polycationic macromolecules, chelating agents and cationic peptides, can bind to polyanionic LPS displacing cross-bridging divalent cations, disorganising the structure of the outer leaflet. This increases permeability of the OM and allows access of lipophilic antimicrobial agents to the phospholipid bilayers. (Vaara 1992).

NPN (1-N-phenyl naphthylamine) is a hydrophobic probe that fluoresces weakly in aqueous environments and strongly when it enters a hydrophobic environment, such as a phospholipid bilayer, allowing detection of changes in permeability (Denyer & Maillard, 2002). It is normally excluded from the hydrophobic core of the OM of Gram-negative bacteria by the closely packed hydrophilic LPS component of the outer leaflet (2000). The chelating agent EDTA destabilises LPS by removal of Ca^{2+} and Mg^{2+}, causing release of LPS and exposure of the inner leaflet of the OM. Polymyxin, a cationic lipopeptide that binds to LPS, similarly destabilises the structure of the outer leaflet and disrupts the cytoplasmic membrane causing lysis and death. When bacteria are treated with EDTA or polymyxin, NPN is able to penetrate the outer leaflet and in the hydrophobic core its fluorescence increases.

Uptake of NPN was used to investigate whether BDM-I is able to rapidly permeabilise and/or directly degrade the OM of *E. coli*. EDTA and polymyxin were used as positive controls.

3.3.2 Materials and Methods

The method was based on that of Helander & Mattila-Sandholm (Anderson *et al.*, 2004; Bellamy *et al.*, 1993; Didenko *et al.*, 2005; Thomas *et al.*, 1999). Mid-log phase *E. coli*, grown in 5 mL MHB at 37°C for 2 h was centrifuged at 3000 ×g 10 min (3K15, Sigma Laborzentrifuge), washed and resuspended in 5 mM HEPES buffer, pH 7.2 (Thermotrace, Vic. AUS) at an OD_{600nm} of 0.5 U. Ten-fold serial dilutions were

plated onto nutrient agar for viable cell counts. The final inoculum for NPN assays was approximately 5×10^6 CFU/well.

The stock solution of BDM-I (100 mg/mL in DMSO) was diluted in 5 mM HEPES buffer to 4× the test concentration of 250 µg/mL and 50 µL added to six wells each of a 96-well flat bottomed microtitre plate (Greiner Bio-One). EDTA (Amresco) and polymyxin B (Sigma-Aldrich) in 5 mM HEPES buffer, pH 7.2 (Thermotrace), were used as a positive controls and added at 4× test concentrations of 250 mM and 250 µM respectively to six well each. A negative uptake control was also included (in triplicate) containing 50 µL HEPES buffer in place of a treatment compound. A stock solution of 50 mM NPN in acetone (BDH chemicals) was diluted in 5 mM HEPES to 40 µM and 50 µL added to half of each group of wells containing test compounds (in triplicate). The remaining group of triplicate wells received 50 µL HEPES buffer. The bacterial suspension 100 µL was added to all test wells excluding the buffer/vehicle control immediately before beginning fluorescence readings. Fluorescence was measured at excitation 350 nm and emission 450 nm within 5 min of addition of bacteria on a multilabel counter (Wallac 1420 Victor^3™, Perkin Elmer, Vic, AUS).

The experiment was repeated twice and the results represented as relative fluorescence values (corrected for background fluorescence in absence of NPN) and NPN uptake values (relative fluorescence values as a ratio of those for the buffer control).

The 'NPN uptake factor' was calculated from the Fluorescence units according to the formula:

NPN uptake factor = (T - C) / (N - B) where:

T = fluorescence of test well containing test compound and NPN,
C = fluorescence of test well containing test compound without NPN
N = fluorescence of well containing buffer and NPN
B = fluorescence of well containing buffer alone.

An increase in fluorescence values is indicative of OM permeability as the hydrophobic compound moves into the hydrophobic core of the OM.

3.3.3 Results

When the OM is disrupted, NPN, which is usually excluded by Gram-negative bacteria, can partition into the hydrophobic core of the OM where it fluoresces strongly. Damage to the OM by an antimicrobial agent will result in enhanced uptake of NPN manifested by fluorescence . NPN uptake by *E. coli* treated with BDM-I, EDTA and polymyxin B is shown in Table 3-1. EDTA and polymyxin B, known OM disrupters, resulted in NPN uptake values 1.5× and 3× that of the negative control containing untreated *E. coli*. Exposure of *E. coli* to BDM-I at 250 µg/mL (≤0.5 × MIC_H) failed to increase fluorescence or NPN uptake values above that of the buffer control (Table 3-1). BDM-I at the highest sub-inhibitory concentration tested did not increase the permeability of the OM of *E. coli*.

Table 3-1. Fluorescence values obtained in an NPN uptake assay using *Escherichia coli* exposed to BDM-I for 5 min.

	Average Relative fluorescence ±SD	Average NPN* Uptake values ±SD
Buffer	2175 ± 371	1.0 ± 0.0
Bacterial suspension	3177 ± 1046	1.5 ± 0.7
+ BDMI 250 µg/mL	2091 ± 665	1.0 ± 0.5
+ EDTA 250 µM	4703 ± 974	2.4 ± 0.2
+ PolyB 0.25 µg/mL	10018 ± 627	4.7 ± 1.1

*NPN uptake values were calculated as described in Section 3.3.2.

3.3.4 Discussion

BDM-I is poorly active against many Gram-negative bacterial species characterised by dense LPS in the OM. Lipophilic antibacterial agents active against Gram-positive bacteria may be unable to cross the OM of Gram-negative bacilli despite a mode of action generally applicable to Gram-negative bacteria. Antimicrobial agents capable of permeabilising the OM by interaction with the LPS component have access to the cytoplasmic membrane and cytoplasm and can allow access to agents otherwise excluded. BDM-I, being uncharged, would not be expected to interact with the predominantly anionic components of LPS in the manner of EDTA and polymyxin. In the short exposure time (5 min) of this experiment BDM-I did not enhance the ability of *E. coli* to take up NPN and therefore did not increase permeability of the outer membrane. Its ability to enter other Gram-negative bacteria with a similar OM structure in effective concentrations suggests it is able to diffuse slowly through the outer leaflet of the cell envelope without damage.

3.4 TRANSMISSION ELECTRON MICROSCOPY OF BDM-I TREATED CELLS

3.4.1 Background

Transmission electron microscopy (TEM) provides useful cytological information about structural and cytoplasmic changes and has been widely used for investigating the effects of antimicrobial agents on microorganisms. Ultrastructural studies can indicate likely targets from the changes produced by an agent to cell structure, flagella, endospores and cytoplasmic organelle organisation. Structural changes can be used to confirm the results of biochemical and cell assays or direct further studies to determine if an effect is a direct or indirect consequence of exposure.

B. cereus and *C. albicans,* strains against which BDM-I is both highly active and microbicidal, were exposed to sub-MIC concentrations of BDM-I and changes in ultramorphology investigated using TEM.

3.4.2 Materials and Methods

Cultures of *Bacillus cereus* and *Candida albicans* were grown overnight in MHB or SLM at 37°C. Each was diluted to McF0.5 in fresh medium and BDM-I in DMSO added to test concentrations of 0.5, 1 and 2 µg/mL for *B. cereus* and 2, 4 and 8 µg/mL for *C. albicans*. Control cultures received the same volume of DMSO. Ten-fold dilutions were prepared and 10 µL plated onto NA for viable counts. Cultures were incubated 24 h at 37°C at 150 rpm. Aliquots (1 mL) of treated culture were transferred to microcentrifuge tubes and pelleted at 4°C for 5 min at 1,180 ×g (3K15, Sigma Laborzentrifugen). Glutaraldehyde (Pro Sci Tech, Qld, AUS), 2.5% (1 mL) in 0.1 M sodium cacodylate buffer (Pro Sci Tech) was run down the side of the tube and agitated for 10 min. Pellets were stored in 2.5% v/v glutaraldehyde overnight where required.

Pellets were washed twice, 15 minutes each, with 1 mL 0.1 M sodium cacodylate buffer. Osmium tetroxide (Pro Sci Tech), 1% (250 µL) in 0.1 M sodium cacodylate buffer was added and left to fix bacteria for 15 minutes. The osmium was removed and pellets washed twice, 15 minutes each, with 500 µL 0.1 M sodium cacodylate buffer. Samples were dehydrated stepwise with ethanol (BDH Chemicals) at 70%, 90% and 100% for 10 minutes each. Final dehydration was with 100% ethanol for 15 minutes. Samples were prepared for infiltration by washing in 1 mL propylene oxide (Pro Sci Tech), 15 min each. Infiltration of the sample was performed on a rotator for 1 h with 1:1 v/v mix of propylene oxide and Procure 812 resin prepared according to manufacturers instructions (Pro Sci Tech). This was replaced with 1:2 v/v mix of propylene oxide and resin for 16 h. Samples were incubated in 100% resin for 2 h before transferring to warmed embedding capsules (size 00, Pro Sci Tech) and replacing with final 100% resin for curing at 60°C for 48 h. Blocks, at room temperature, were trimmed and sections cut using an ultramicrotome (Ultracut, Leica Microsystems, NSW, AUS, formerly Reichert-Jung) with a freshly prepared glass knife. Sections were collected on 200 µm copper grids and stained with 2% uranyl acetate for 20 minutes, washed in CO_2-free water and stained with lead citrate 10 minutes. Sections were viewed at 80 kV on a transmission electron microscope (100 SX, JEOL, Tokyo, Japan). Three sections per specimen were examined by TEM on different magnifications in at least five fields of view and photographs taken on film and developed on site.

3.4.3 Results

Sections of BDM-I treated cultures of *Bacillus cereus* and *Candida. albicans* were examined by TEM for morphological changes and compared to untreated cultures. Treated bacterial and yeast cells were comparable to untreated cells with

respect to the features of cell morphology that could be observed with the achieved magnification and resolution (Figure 3-3 and Figure 3-4).

In *Bacillus cereus* there was no evidence of swollen cells, thickened, incomplete or defective walls, abnormal septation or detached or undulating cytoplasmic membranes in BDM-I-treated cells (Figure 3-3). The resolution was not adequate for detection of changes in sub-cellular structures, however, cytoplasmic aggregates or uneven staining were not observed. The only notable effect was the lower incidence of endospores in treated samples compared to control samples. *C. albicans* treated cells appeared morphologically similar to control cells and showed normal budding.

3.4.4 Discussion

The action of BDM-I at sub-lethal concentrations on bacterial and yeast cells does not result in morphological changes in the cell wall or cytoplasmic membrane or gross changes in the cytoplasmic contents. Many reports in the literature show changes in ultramorphology in bacteria and yeasts as a result of exposure to agents acting on the cell-wall and cell-membrane (Errington, 2003). The absence of morphological changes confirms the results of the cell assays reported in this chapter and indicates that BDM-I is not affecting the integrity of the cell wall or cell membrane of bacterial and yeast cells.

Exposure to BDM-I appears to lower the incidence of endopsore formation in *Bacillus cereus*. Sporulation is a very complex response to stresses such as starvation and high cell density and involves coordinated monitoring of many internal and external signals channelled through separate regulatory systems (Errington, 2003). The

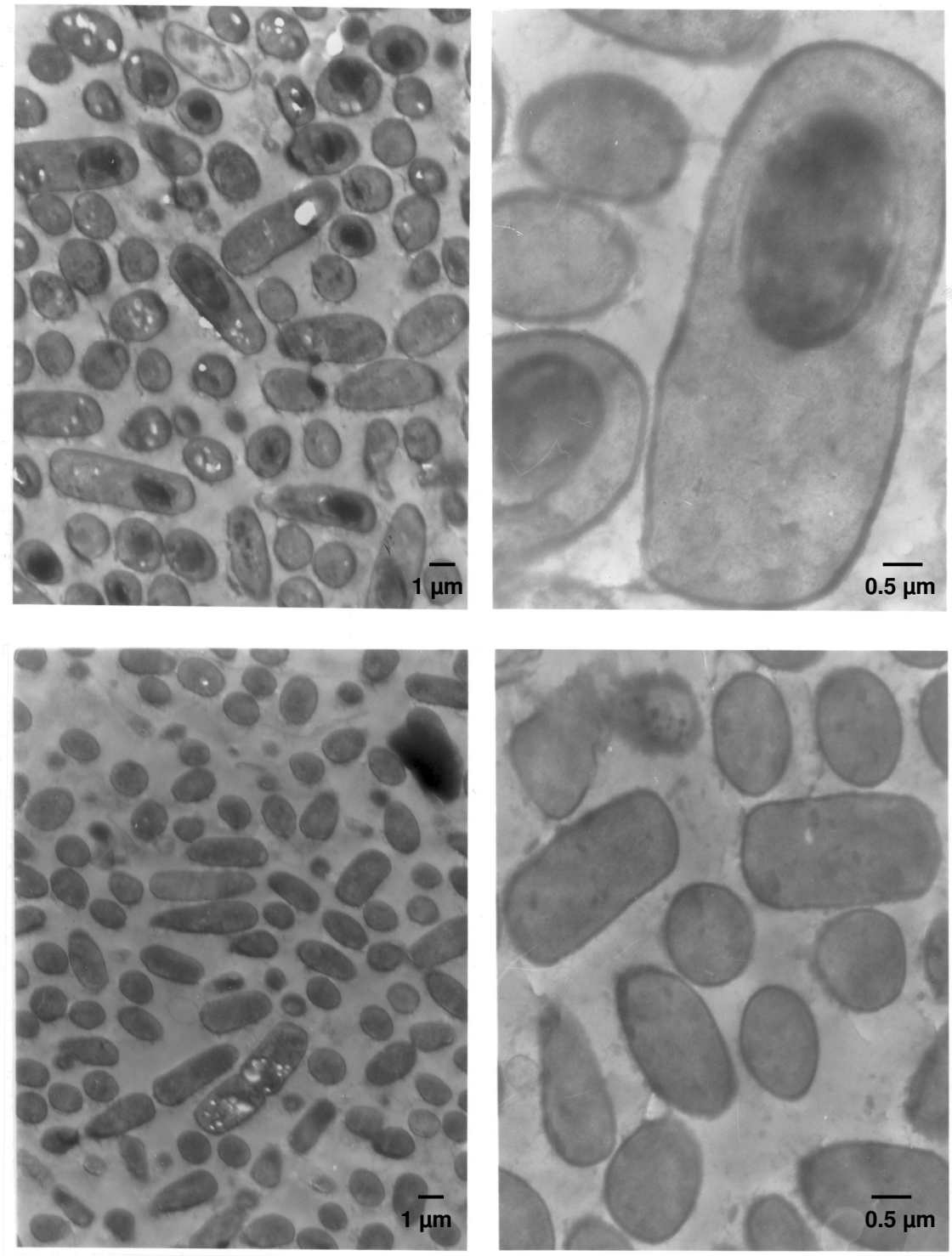


Figure 3-3. Transmission electron micrograph of *Bacillus cereus* treated with BDM-I.

B. cereus untreated control (top right, top left) and *B. cereus* treated with 2 µg/mL BDM-I for 24 h (Bottom left, bottom right).

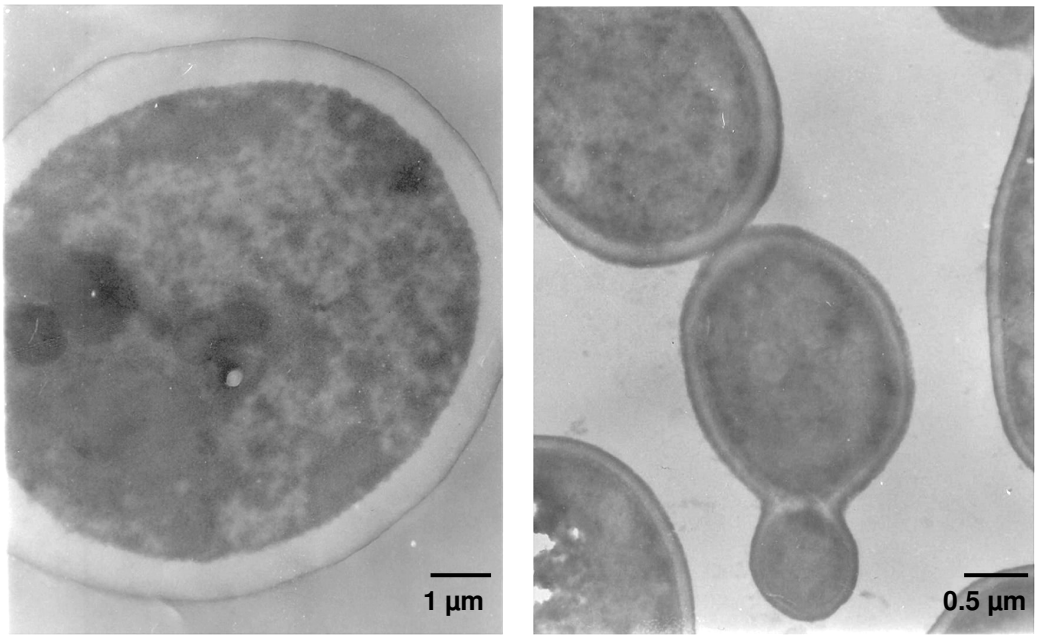


Figure 3-4. Transmission electron micrograph of *Candida albicans* treated with BDM-I.

C. albicans untreated control (left) and treated with 4 µg/mL BDM-I for 24 h (right).

formation of endospores has been extensively studied in *B. subtilis* and shown to involve complex regulatory systems. The most prominent transcriptional regulator is Spo0A, whose synthesis is controlled transcriptionally and whose activity is regulated by phosphorylation (Iber *et al.*, 2006). Phosphorylated Spo0A is an essential positive regulator of sporulation, activating transcription of several sporulation-specific genes, *spoIIAB*, *spoIIAA* and *spoIIE* (Jiang *et al.*, 2000). Spo0A is phosphorylated on aspartate by a cognate histidine kinase, Spo0B, which is part of a phosphorelay signal transduction system transferring phosphates ultimately to Spo0A and controlling the initiation of sporulation. Multiple kinases in this system provide signal inputs with ultimate transfer to Spo0A. Negative regulation occurs through controlled dephosphorylation by aspartyl phosphate phosphatases, such as the RapA and RapB phosphatases which dephosphorylate Spo0A (Proctor *et al.*, 1998). To date, tyrosine-phosphorylated proteins have not been shown to play a role in the initiation, or in the

inhibition of initiation, of sporulation or in the various stages of cell differentiation leading to spore formation. Many different signals are integrated in determining sporulation and very little is yet known about many aspects signal transduction in bacteria. Sporulation can be inhibited at many stages of endospore formation, such as asymmetric cell division, chromosome segregation, engulfment of the pre-spore and spore morphogenesis. Further work is required to determine whether BDM-I does inhibit endospore formation in *Bacillus* spp. and, if so, which of the many complex components is affected.

The results of the microarray analysis of gene transcription in BDM-I treated cells of *B. subtilis* reported in Chapter 6 have shown a change in genes involved in the sporulation process which supports the observation that BDM-I has inhibited sporulation in the closely related *B. cereus.* The implications of this for the mechanism(s) of action of BDM-I remains to be explored.

CHAPTER 4 EFFECT OF BDM-I ON COLONIAL MORPHOLOGY AND CELL PHYSIOLOGY

4.1 INTRODUCTION

In search of clues to BDM-I targets in bacteria, physiological changes in response to BDM-I in individual species were examined. Species-specific physiological effects (in colony size, pigment, motility) that had been observed in response to exposure to BDM-I were investigated. These changes had been observed during MIC and MMC assays and included the formation of small colony variants by *S. aureus*, inhibition of pigment formation by *Serratia marcescens* and inhibition of swarming motility in *Proteus* spp. These were examined more closely along with capsule formation by *Klebsiella pneumoniae* as this is an important virulence factor for many bacteria.

4.2 INDUCTION OF SMALL COLONY VARIANTS OF *S. AUREUS*

4.2.1 Background

Small-colony variants (SCV's) of *Staphylococcus aureus* are a slow-growing heterogeneous sub-population of colonies, generally ten times smaller than the 'normal' phenotype that cause major problems in persistent and recurring infections (Proctor *et al.*, 1995). They may be a naturally occurring part of a population and can be induced following exposure to aminoglycosides, β-lactams or sulphonamide-trimethoprim (Baumert *et al.*, 2002; Proctor *et al.*, 1994). These colony variants are non-pigmented and non-haemolytic with reduced metabolic capabilities. This leads to a

number of physiological changes, including reduced production of virulence factors such as coagulase and α toxin and increased resistance to antibiotics, such as aminoglycosides and penicillins (Proctor *et al.*, 1995). There are possibly several mutations leading to the small colony variant phenotype that have not been well characterised, since colony stability varies considerably and revertants to normal morphology are common (Baumert *et al.*, 2002).

Many SCV's are respiratory deficient and have been shown to be auxotrophic for menadione, thiamine and haemin, important precursors for, respectively, menaquinone and the cytochromes, electron acceptors in the electron transport chain required for oxidative phosphorylation (Baumert *et al.*, 2002). Reduced electron transport, and consequential lack of ATP, results in many phenotypic changes such as the small colony size, reduction in biosynthesis of carotenoids and reduced secretion of macromolecules. A reduced membrane potential in small colony variants is also responsible for an increased resistance to antibiotics that require a high electrochemical gradient for efficient uptake such as the aminoglycosides (Dierstein *et al.*, 1989).

The production of variable sized colonies was noted in MMC assays for *S. aureus.* The production and stability of small colony variants on exposure to BDM-I was investigated in *S. aureus* since their development could indicate induction of mutations or selection of existing stable small colony phenotypes with genetic or biochemical lesions useful for studies of MOA.

4.2.2 Materials and Methods

MHB (5 mL) containing 0, 32 or 128 µg/mL BDM-I with a final DMSO concentration of 1% v/v, was inoculated with 100 µL log phase culture of *S. aureus* in 5 mL MHB, visually standardised to 10^5-10^6 CFU/mL by McFarland opacity standards

and the cultures incubated at 30°C, aerobically to improve pigment production (Denisenko *et al.*, 27 Dec. 2002 International patent, Nicoletti et al., unpublished).

Aliquots of 100 µL from test and control cultures were taken at 0, 24 and 48 h after inoculation and serially diluted ten-fold and plated onto NA and incubated aerobically for up to 48 h at 37°C. Dilution plates were observed for colony morphology and characteristics. Selected colonies from dilution plates showing variations in size and pigment were confirmed by Gram-stain and catalase production (H_2O_2 slide test). Typical variant colonies (3-5 per plate) from 24 and 48 h exposures were serially subcultured onto BDM-I-free nutrient agar to test for phenotypic stability. The assay was repeated twice and plates photographed with a digital camera (A520, Canon).

4.2.3 Results

BDM-I at 32 and 128 µg/mL is not microbicidal to *S. aureus* at 24 and 48 h and a sufficient number of colonies were produced on dilution plates of concentrations up to 512 µg/mL to be able to observe for colony heterogeneity. Colonies of *S. aureus* grown in MHB and 1% DMSO had uniform colony diameters of 2 mm (Figure 4-1, right) when plated on NA. Twenty four or 48 h exposure to BDM-I produced either uniform small colonies of ~1 mm diameter (Figure 4-1, left) or colonies of variable size from normal to small (data not shown). Colonies showed loss of pigment but were otherwise of typical staphylococcal morphology. Gram-reaction and cell morphology for variant colonies was the same as control cells and all were catalase positive. On subculture to NA, small colonies from both concentrations and both times gave rise to heterogeneous colonies (normal to small) at either the first or second subculture. No stable variant phenotypes resulted from exposure to concentrations of BDM-I for up to 48 h at concentrations up to 128× the MIC. After subculture in drug-free medium

variant colonies had the same MIC as the parent. No further characterisation of atypical colonies was performed. BDM-I has not, in this experiment, produced any mutant colonies or the stable small colony variants noted with other antibiotics.

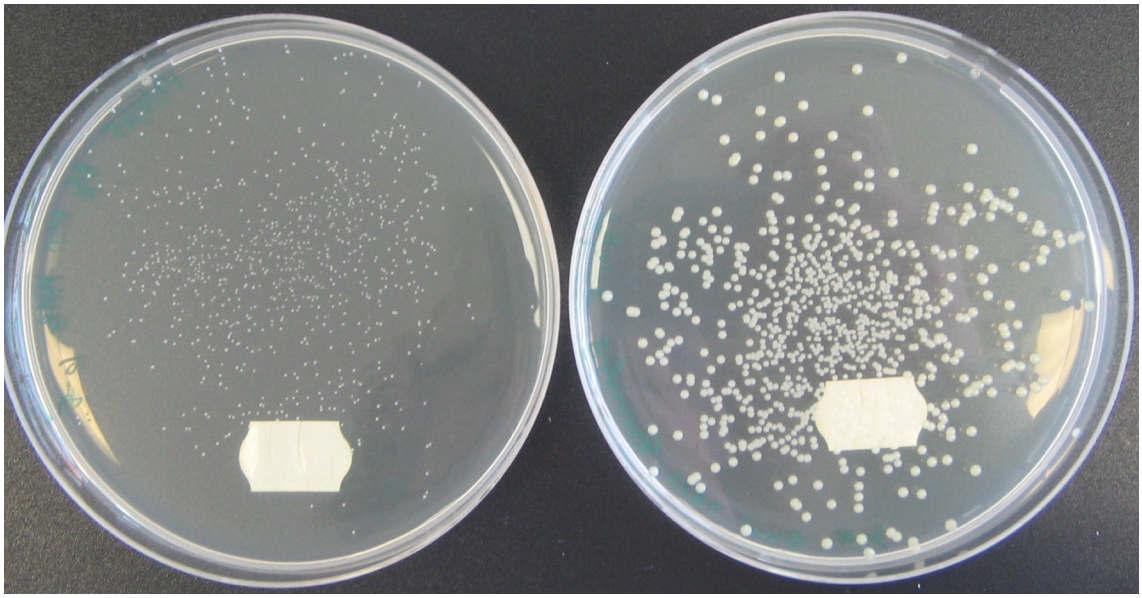


Figure 4-1. Small colonies of *Staphylococcus aureus* produced on agar after exposure to BDM-I in broth culture.

S. aureus was exposed to 32 µg/mL BDM-I for 24 h in broth, plated out (left) and compared to a control culture (right).

4.2.4 Discussion

Exposure of *S. aureus* to BDM-I for up to 48 h did not induce or select any resistant mutant colonies, or the stable small colony variant phenotype in *S. aureus* which occur on exposure to antibiotics such as β-lactams and aminoglycosides. BDM-I has been shown to be non-mutagenic by the *Salmonella* mutation reversion (Ames) test. (Nicoletti *et al.*, unpublished). No resistant mutants were observed during 12-16 week continuous exposure to BDM-I of *S. aureus* and several other bacterial species in resistance studies (Kennelly, 2002). Very low incidence of stable resistant phenotypes is consistent with a single major target that cannot be readily circumvented by bacteria.

Lack of development of resistant phenotypes on exposure to BDM-I is also consistent with a target such as inhibition of bacterial PTP's. PTP's all share the same catalytic site but have many different functions which may vary between species (1975). Inhibiting bacterial phosphatases may be as effective as targeting multiple enzymes within a cell.

On the evidence to date it is highly unlikely that BDM-I affects respiratory energy metabolism (Section 2.1). The lack of an effect of BDM-I on ATP synthesis is supported by evidence in Chapter 5. Stable phenotypes with genetic and/or metabolic lesions would have been useful for investigations of the biochemical effects of BDM-I. However, it is important to know that exposure to BDM-I does not induce slow growing stable and resistant variants that could survive treatment concentrations and cause persistent infections. Small unstable colonies of *E. faecalis* and *B. subtilis* were also observed after exposure to BDM-I, however production of these variants was not as easily reproduced as for *S. aureus* (data not shown).

4.3 PRODIGIOSIN PRODUCTION IN *SERRATIA MARCESCENS*

4.3.1 Background

Serratia marcescens is a plant and insect pathogen and an opportunistic human pathogen that is often multi-drug resistant and causes nosocomial respiratory and urinary tract infections. It is one of three *Serratia* spp. that can produce an unusual, cell-associated intense red pigment termed 'prodigiosin' by Gerber (Boger & Patel, 1988). Prodigiosin (2-methyl-3-pentyl-6-methoxy prodigiosin) is one of a number of red pigments, based on the characteristic pyrrolylpyrromethene skeleton, referred to as prodigiosins or prodiginenes (Williamson *et al.*, 2006). Prodiginenes are also produced by *Streptomyces* spp. and various marine bacteria (Fineran *et al.*, 2007; Montaner *et al.*,

2000; Perez-Tomas *et al.*, 2003; Thomson *et al.*, 2000). Serratia also produce an antibiotic, carbapenem and exo-enzymes such as pectinases, proteases and cellulases.

Prodigiosin (PG) is a secondary metabolite with no known role in the physiology of producing strains but reported to have antimicrobial (antifungal, antibacterial and antiprotozoal), immunosuppressant and anti-cancer activity (Harris *et al.*, 2004). There is considerable heterogeneity among *Serratia* strains with some non-prodigiosin producing species possessing 'cryptic' genes for PG production while others possess none (Carbonell *et al.*, 2000). PG production is characteristic of environmental strains of *S. marcescens* and clinical isolates are usually non-pigmented (Ang-Küçüker *et al.*, 1999). Non-pigmented variants, while proving more invasive in the conjunctival sacs of guinea pigs (Serenty test), have much lower LD_{50} values in mice than pigmented variants (Fineran *et al.*, 2007). A mutant strain of *S. marcescens* deficient in a protein (PigX) that represses transcription of the PG biosynthetic operon (*pigA-pigO*), exhibited increased production of PG and degradative exo-enzymes and increased plant virulence (Williamson *et al.*, 2006). Regulation of the swarming phenotype in *Serratia* was also shown to involve *pigX*. PG, through its broad antibiotic action, may play a role in the competitive survival of *Serratia.*

Synthesis of PG occurs as result of multiple cellular and environmental cues, a situation which is true for many secondary metabolites and necessitates complex genetic regulation of biosynthesis. Investigations to date have shown regulatory variation between strains of the same genus and between genera (Fineran *et al.*, 2007). The PG biosynthetic pathway in *Serratia* has been described and the complex regulatory network for PG production elucidated (Williamson *et al.*, 2006). In *Serratia*, the *pig* operon containing genes *pigA-pigO* has been fully identified and specific Pig enzymes assigned to steps in biosynthesis (Fineran *et al.*, 2007). Regulator genes reverse the repression of transcription of *pig* biosynthetic genes in response to cell

signalling. A master regulator, *pigP*, negatively regulates expression of the *pig* operon by modulating expression of other regulators, repressing both pigment and exoenzyme production (Whitehead *et al.*, 2001).

Bacteria regulate the induction and repression of genes in response to environmental cues, such as the density of the bacterial cell population, by extracellular signalling or quorum sensing (QS) systems. Cell density-dependent gene regulation is mediated by the production of small signalling molecules, such as *N*-acyl homoserine lactones (AHL's) in Gram-negative bacteria. These small molecules accumulate and, above a threshold density, act as autoinducers of genes in the producer organisms. This process enables populations of bacteria to cooperate to achieve particular physiological outcomes which optimise growth or survival in their environment (Eberl *et al.*, 1999; Morohoshi *et al.*, 2007). QS regulates the expression of virulence factors in many pathogens. QS signalling in *Serratia* controls antibiotic and pigment production, biofilm formation and virulence factors such as the secretion of degradative exoenzymes and a surfactant (serrawettin) which facilitates swarming motility (2000). Thomson *et al.* (Blizzard & Peterson, 1963; Williams & Gott, 1964) showed that carbapenem, PG and degradative exoenzyme production was mediated by QS AHL's which derepress transcription of the PG biosynthetic genes by modulating expression of regulator genes.

Inhibition of PG production in *Serratia* spp. by antibiotics has been reported. Streptomycin, tetracycline and chloramphenicol inhibit PG biosynthesis by interference with synthesis of pigment precursors (Tsang & Feng, 1983). Polymyxin B inhibits PG production secondary to damage to the outer membrane (1999). Ang-Küçüker *et al.* (Dierstein *et al.*, 1989) report inhibition of PG by subinhibitory concentrations of erythromycin, tobramycin, imipenam, co-trimoxazole, cefoxitin and nitrofurantoin. The effect of BDM-I on prodigiosin synthesis and secretion in *Serratia*

marcescens was investigated following the observation of a loss of pigment in colonies in MMC assays for BDM-I.

4.3.2 Materials and Methods

Prodigiosin production in *Serratia marcescens* was evaluated by modification of methods used by (Williamson *et al.*, 2006). *S. marcescens* (RMIT 342/1-32) was grown for 6 h in 30 mL peptone glycerol (PGB) broth aerobically at 37°C and on a shaker (Ratek) at 160 rpm. The culture was centrifuged 10 minutes at 5000 ×g to concentrate cells (3K15, Sigma Laborzentrifugen). Cells were resuspended in 5 mL sterile distilled water and stored overnight at room temperature as inoculum for tests.

The stock cell suspension was diluted in PGB to an OD_{660nm} 0.05 as the starting inoculum. This culture was divided into four aliquots of 15 mL in 50 mL culture bottles. BDM-I (15 µL) was added at 8, 32 and 128 µg/mL from 100× stock solutions in DMSO (BDH chemicals). The control received an equal volume of DMSO. All cultures were incubated aerobically at 30°C, 160 rpm, for up to 48 h. At the time of BDM-I addition (0 h) and at 4, 8 and 12 h, 1.5 mL was removed from each culture and OD_{660nm} used to to monitor cell density. An aliquot of 100 µL was also removed at each time point and 10-fold dilutions (10 µL) plated onto NA for viable counts to monitor cell density. Plates were incubated for 24 h at 37°C. Pigment production in broth cultures was recorded at 0, 4, 8 and 12 h with a digital camera (A520, Canon).

An aliquot of 300 µL was removed from all test and control samples into 2.7 mL methanol with 4% 1 M HCl and left at room temperature for 1 h to extract the pigment. The solution was then clarified by centrifugation at 5000 ×g for 15 min and the OD_{500nm} used to detect the extracted pigment. Percentage inhibition of pigment production was calculated for each treatment and time point by the formula:

$((OD_{ctrl}-OD_{test})/OD_{ctrl}) \times 100$. Qualitative and quantitative assays were repeated three times on different days.

4.3.3 Results

Serratia marcescens was exposed to BDM-I in PGB and development of pigment formation recorded visually (Figure 4-2) and spectrophotometrically (Figure 4-3). Maximum inhibition of pigment production occurred at 8 h. There was no marked dose-response, 8 μg/mL being almost as effective as 128 μg/mL at 8 h (Figure 4-3). Cell density, monitored by viable counts, showed that there was insignificant inhibition of *S. marcescens* at 128 μg/mL so the effect on pigment production was not a result of lower cell numbers. BDM-I inhibits prodigiosin production at a concentration >64-fold less than its MIC for *Serratia.*

4.3.4 Discussion

Inhibition of pigment formation by *Serratia marcescens* was evident in the presence of BDM-I at concentrations well below the MIC. Strains producing prodiginenes inhabit very diverse environments and belong to diverse bacterial genera. Secondary metabolites such as these show a great molecular diversity with specific functions of most not yet identified. Genetic regulation of the production of prodiginenes which must integrate multiple cellular and environmental signals and therefore varies considerably between strains of the same genus and between genera (Williamson *et al.*, 2006).

Pigment production has been linked to protein phosphorylation in bacteria. Phosphorylation of PigQ, one of the pig response regulators, results in activation of prodigiosin biosynthesis by transcription of the *pig* operon (Matsumoto *et al.*, 1994; Umeyama *et al.*, 2002). In *Streptomycess coelicolor,* phosphorylation on serine and

threonine residues of AfsR, a transcriptional factor in the regulation of secondary metabolism and morphological differentiation, is one factor controlling pigment production (1996). Li & Strohl (Umeyama *et al.*, 1996) isolated a gene, *ptpA*, from *Streptomyces coelicolor* coding for a low molecular weight tyrosine phosphatase (LMWPTP). Cloned into *Streptomyces lividans, ptpA* resulted in production of undecylprodigiosin and a pigmented antibiotic actinorhodin (Li & Strohl, 1996). PtpA belongs to a family of highly conserved small acidic PTP's (SA-PTP) in bacteria, fungi and mammalian tissues. Protein sequencing shows that PtpA is more closely related to mammalian and yeast SA-PTP's than to bacterial SA-PTP's (2004). Bacterial SA-PTP's are clustered with different types of genes in different species. The less closely related SA-PTP's in *Erwinia amylovora, Pseudomonas solanacearum and Klebsiella pneumoniae* are associated with exopolysaccharide synthesis.

Fürstner *et al.* (Harshey, 2003) have shown that nonylprodigiosin from *Streptomyces,* is an inhibitor of PTP's and DSP's. Like BDM-I, prodigiosin was less inhibitory to DSPs. PTP inhibition is a possible explanation of the antibiotic action of prodigiosin. Inhibition of a PTP similar to PtpA of *Streptomyces* could be the cause of the inhibition of prodigiosin production in *Serratia* noted here for BDM-I. It would be interesting to test the effect of combinations of BDM-I and PG for synergistic or antagonistic inhibition of bacteria, given that they may share a general mode of action.

The proposed MOA of BDM-I is inhibition of bacterial tyrosine phosphatases (Chapter 5). This action may explain the observed suppression of pigment production.

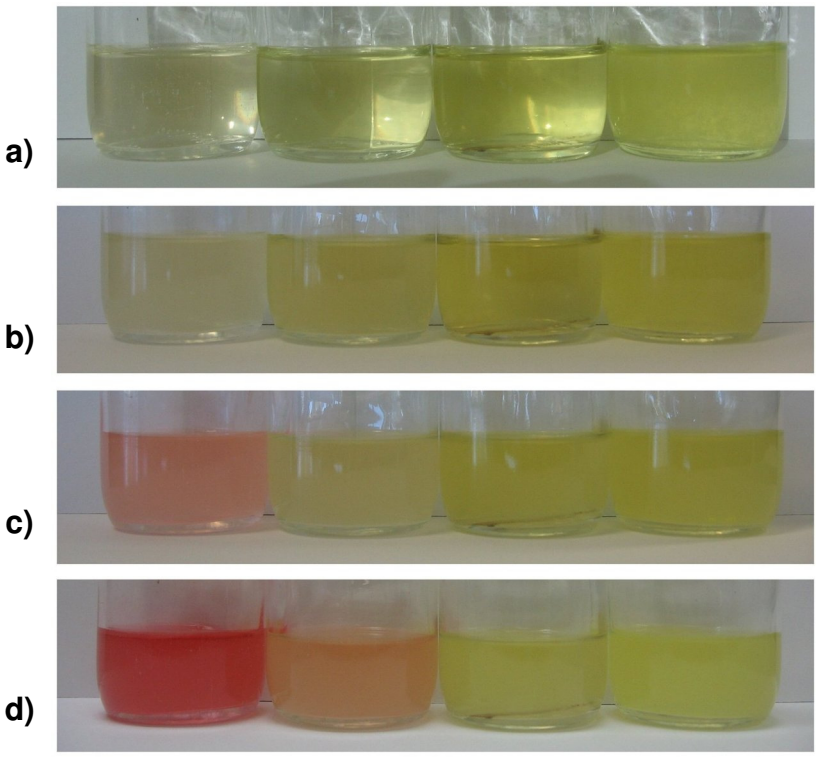


Figure 4-2. Inhibition of pigment production in *Serratia marcescens* by BDM-I.

BDM-I concentrations in broth (left to right): 0, 8, 32, 128 µg/mL. Exposure times were 0 h (a), 4 h (b), 8 h (c), 12 h (d).

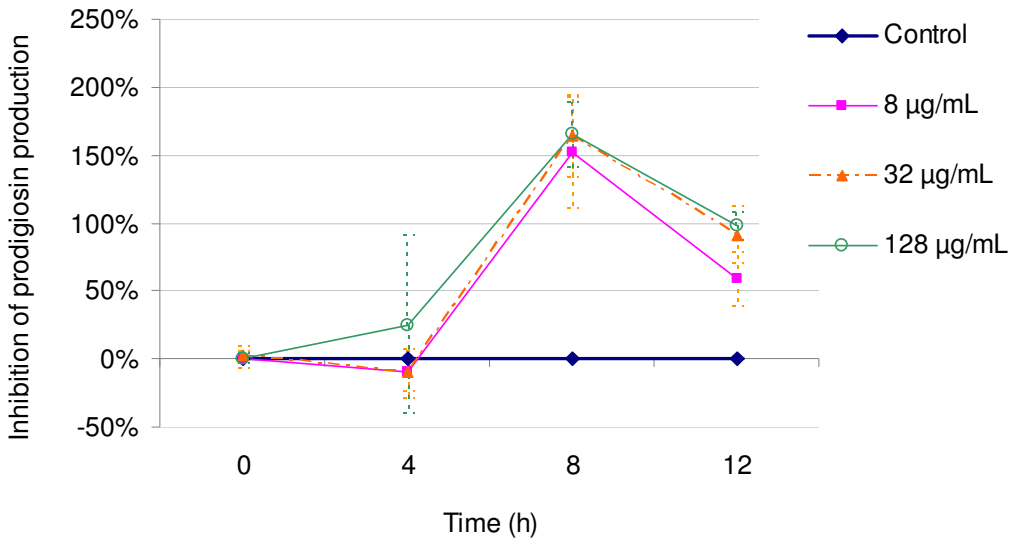


Figure 4-3. Inhibition of pigment production by *Serratia marcescens* exposed to BDM-I measured by absorbance of prodigiosin extracts.

OD_{500nm} measurements used to calculate inhibition of prodigiosin as described in Section 4.3.2. Dotted vertical bars represent standard deviation.

4.4 SWARMING MOTILITY IN *PROTEUS* SPP.

4.4.1 Background

Many bacteria are capable of self-propelled motion in certain circumstances and this motility can be achieved by a variety of mechanisms. They may be driven by flagella or utilise a variety of flagella-independent surface translocation mechanisms such as gliding (Harshey, 2003). The term swarming describes a unique type of flagella-dependent surface translocation involving the coordinated and rapid movement of bacteria across a semisolid surface and facilitating rapid colonisation, attachment to surfaces and biofilm formation. Many bacterial species are motile but do not necessarily swarm. Though both are dependent on flagella, swarming of a group of bacteria differs from the 'swimming' motility of individual bacterial cells through liquid. Genera of flagellated bacteria known to be capable of swarming include *Bacillus*, *Escherichia*, *Salmonella*, *Serratia, Pseudomonas*, *Vibrio, Yersinia* and *Proteus* (Lai *et al.*, 1998).

Cells at the edge of a swarming colony differentiate into specialised elongated, multinucleated 'swarmer' cells, possessing up to 50 times as many flagella (Eberl *et al.*, 1999). Swarmer cells continue to move outwards as a coordinated group, while cells inside the colony continue to replicate (Allison & Hughes, 1991). For *Proteus*, a characteristic periodic pattern of concentric circles is produced from the point of inoculation due to the repetitive alternation between growth of stationary cells (consolidation) and the production and subsequent migration of swarmer cells (Eberl *et al.*, 1999).

Swarming of cells is dependent on factors such as high nutrient availability, surface viscosity, wettability, lowered surface tension and cell density. Secretion of exopolysaccharides and peptide surfactants facilitates swarming in *Serratia* (Gygi *et al.*,

1995). *Proteus mirabilis* does not secrete surfactant peptides but secretes a capsular polypeptide that reduces surface friction (Ariison *et al.*, 1992; Davies *et al.*, 1998; Iwalokun *et al.*, 2004; Liaw *et al.*, 2000; Young *et al.*, 1999). Swarming motility in *Proteus* spp. has been associated with colonisation, biofilm formation and the expression of virulence factors including protease, urease hemolysins and exopolysaccharides (Armitage, 1981). Cells have also been found to have altered metabolic activity during swarming, including reduced rates of protein and nucleic acid synthesis and oxygen uptake (Eberl *et al.*, 1999).

Swarming is a coordinated, social behaviour where bacterial communication is essential and quorum sensing is an important regulatory mechanism. The two coordinating pathways involved in the regulation of swarming motility are reviewed by (Liu & Matsumura, 1994). Flagellar regulator genes control cell elongation and expression of flagella, and a QS signalling system based on AHL's controls synthesis of extracellular molecules essential to cell-to-cell interactions. In *E. coli* and *S. typhimurium* the flhDC flagellar operon encodes transcriptional regulators (FlhD and FlhC) that control the expression of genes related to flagellar structure, chemotaxis and cell division (Furness *et al.*, 1997; Tolker-Nielsen *et al.*, 2000). Expression of flagellar operon, flhDC, mRNA in *P. mirabilis* is 30-fold higher in swarm cells than in vegetative cells (Williams & Schwarzhoff, 1978).

A number of antibiotics have been shown to inhibit swarming including neomycin, sulfonamides and combinations of polymyxin with chloral hydrate or sodium azide (Hussain *et al.*, 1998). These antibiotics have different mechanisms of action and may affect different aspects of the complex pathways controlling swarming behaviour. The most well known and efficient specific inhibitor of swarming, ρ-nitrophenylglycerol (PNPG), is not an antibiotic and prevents swarming by inhibition of differentiation into swarm cells (Logan *et al.*, 1989).

Phosphorylated serine residues have been found in *Campylobacter* flagellin protein (Kelly-Wintenberg *et al.*, 1993) and phosphorylated tyrosine residues in type a and b *Pseudomonas* flagellin proteins (Kelly-Wintenberg *et al.*, 1993). A membrane-bound tyrosine kinase has also been reported (Liaw *et al.*, 2000) and though their functions are not yet clear, it may be valuable structurally or related to flagellar assembly or transport.

It was decided to investigate the effect of BDM-I on swarming in *Proteus* spp. since some inhibition of swarming by *Proteus vulgaris* had been noted during BDM-I time-kill studies while no inhibition of flagellar swimming motility was evident using a semisolid agar assay (Nicoletti *et al*., unpublished).

4.4.2 Materials and Methods

Exposure to BDM-I during surface growth

Luria broth agar (LBA) plates were prepared based on the method of (Liaw *et al.*, 2000; Liaw *et al.*, 2001) containing 1% agar to facilitate swarming of *P. mirabilis* and *P. vulgaris*. BDM-I in DMSO was added to the molten agar immediately before pouring to give two-fold concentrations of BDM-I between 4 and 64 µg/mL and a DMSO concentration of 1% v/v. All plates were made the week of testing, stored at 4°C and dried at 37°C for 30 minutes before inoculation.

A colony from a fresh overnight plate of each species was used to inoculate 5 mL MHB and incubated aerobically for 6 h at 37°C, on a shaker (Ratek) at 160 rpm. The culture, 5 µL, was used to centrally inoculate agar plates containing BDM-I at 4, 8, 16, 3 and 64 µg/mL. Plates were incubated aerobically at 37°C for 16 h.

Exposure to BDM-I prior to surface growth

LBA plates were prepared as above without the addition of BDM-I. A colony from a fresh overnight plate of each species was used to inoculate 5 mL MHB containing BDM-I at 8, 32 and 128 μg/mL and incubated aerobically for 6 or 24 h at 37°C on a shaker (Ratek) at 160 rpm. At each time point, LBA plates were inoculated centrally with 5 μL of the overnight culture and incubated aerobically at 37°C for 16 h.

Both assay methods were completed twice. For both exposure methods, confluency of growth at the inoculation point, indicating cell viability and patterns of growth from the centre point were recorded and plates photographed with a digital camera (A520, Canon).

4.4.3 Results

Exposure to BDM-I during surface growth

P. mirabilis and *P. vulgaris*, grown in drug free MHB for 6 h, were centrally inoculated onto an LBA plate containing BDM-I to demonstrate a direct effect on characteristic swarming patterns during surface growth. *P. vulgaris* and *P. mirabilis* grew as a confluent central button on LBA at 64 μg/mL, indicating the MIC in LBA by agar dilution was > 64 μg/mL, a concentration ≥6-fold higher for *P. vulgaris* than the mean MIC in MHB (11 μg/mL) (Figure 4-4a). The higher MIC in LBA is not surprising since medium components affect the MIC. BDM-I has previously been found to bind to agar and therefore the effective concentration may be less than the concentration added to the agar (Nicoletti *et al*., unpublished).

BDM-I caused dose dependent inhibition of swarming in both *P. mirabilis* and *P. vulgaris* at a concentration below that causing whole cell toxicity in the test medium (Figure 4-4). There was complete inhibition of swarming of *P .mirabilis* by 64 µg/mL and of *P. vulgaris* by 32 µg/mL BDM-I. Inhibition of swarming in *P. mirabilis* occurred at a much lower concentration relative to its MIC in MHB than was the case with *P. vulgaris*. The lesser inhibition of swarming in *P. vulgaris* seen for the concentration of 32 µg/mL when BDM-I is incorporated in LBA (Figure 4-4a) is explained by the interference of medium components and agar resulting in lower effective concentration of BDM-I.

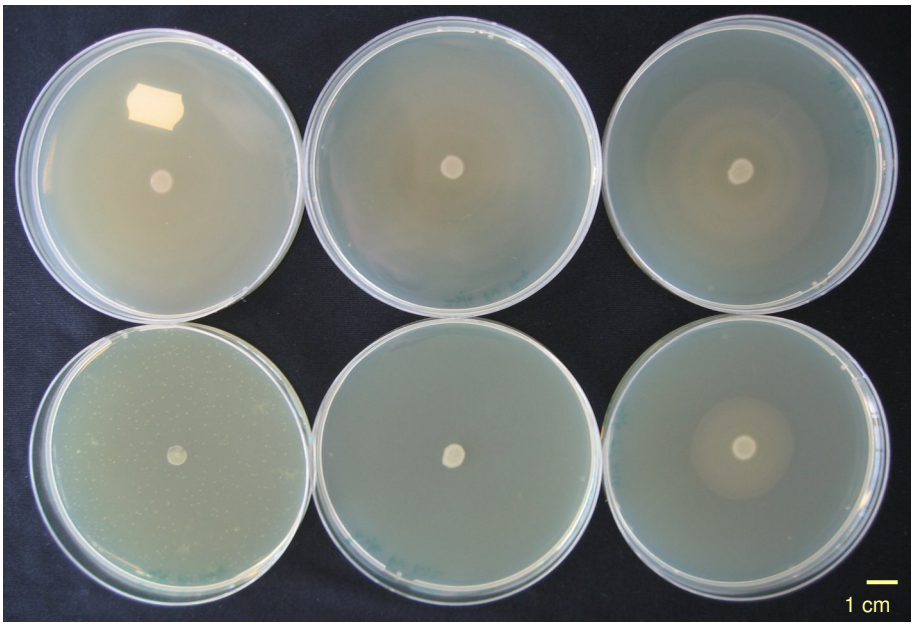
Exposure to BDM-I prior to surface growth

Pre-exposure of *P. vulgaris* to 8, 32 and 128 µg/mL BDM-I in MHB for 6 h or 24 h before plating on LBA gave a dose dependent inhibition of swarming at both times, the inhibition being greater after 24 h exposure (Figure 4-5). Swarming was almost completely inhibited by pre-exposure to 32 µg/mL for as little as 6 h.

Pre-exposure to BDM-I for 6 h did not affect cell viability of *P. mirabilis* and all treated cultures swarmed on LBA plates to the same extent as the control (results not shown). Growth of *P. mirabilis* after 24 h pre-exposure to 128 µg/mL BDM-I showed only a few colonies at the point of inoculation (See arrow in Figure 4-6). Swarming was completely inhibited in these few colonies surviving exposure to 128 µg/mL. The concentration range used failed to show a dose response for *P. mirabilis*.

Exposure to BDM-I for 6 h has irreversibly affected the ability *P. vulgaris* to swarm on LBA, in a manner similar to when it is continuously present in LBA during growth. A similar irreversible inhibition for *P. mirabilis* occurred only after exposure to 128 mg/mL for 24 h.

a)



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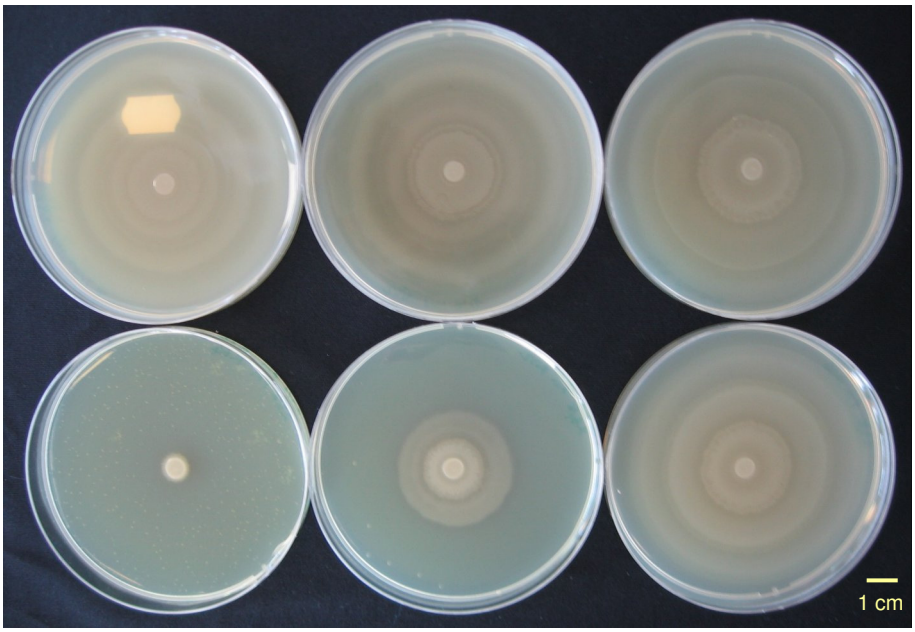
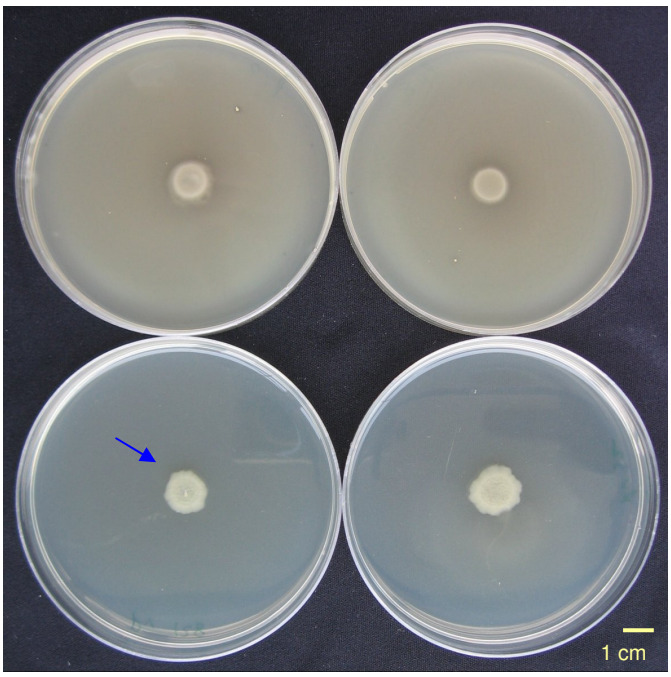


Figure 4-4. Swarming assays of *Proteus vulgaris* and *Proteus mirabilis* on agar containing BDM-I.

P. vulgaris (a) and *P. mirabilis* (b) cultures (5 µL) were grown in MHB for 6 h and used to centrally inoculate LBA containing 0, 4, 8, 16, 32, 64 µg/mL BDM-I (clockwise from top left in each photograph).

a)



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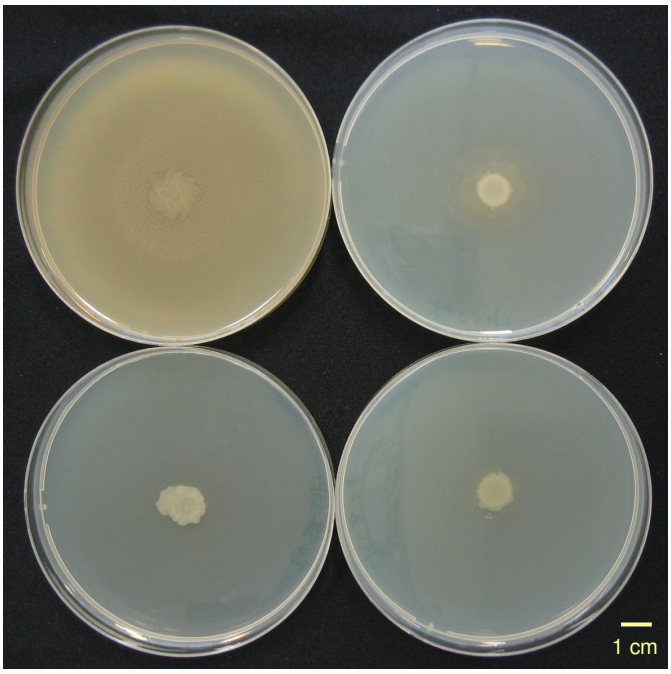


Figure 4-5. Surface swarming of *Proteus vulgaris* exposed to BDM-I in broth before plating onto BDM-I free LBA.

Concentrations of BDM-I during broth exposure for 6 h (a) and 24 h (b). BDM-I exposure concentrations clockwise from top left in each photograph: 0, 8, 32, 128 µg/mL. Blue arrow in a) indicates inhibition of swarming without inhibition of growth at the inoculation point.

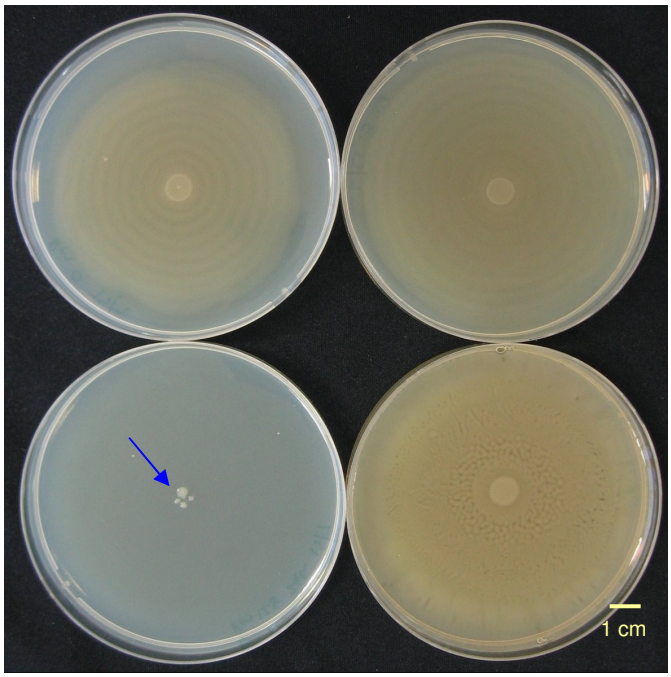


Figure 4-6. Surface swarming of *Proteus mirabilis* exposed to BDM-I in broth prior to growth on BDM-I-free LBA.

Concentrations of BDM-I, clockwise from top left, 0, 8, 32, 128 µg/mL. Blue arrow indicates inhibition of growth at the inoculation point. (Six hour exposure data not shown).

4.4.4 Discussion

BDM-I inhibited swarming in *Proteus mirabilis* at 64 µg/mL and in *P. vulgaris*, at 32 µg/mL when BDM-I was continuously present in the medium during surface growth. Whole cell toxicity, as measured by standard MIC in MHB, is 128 µg/mL and 11 µg/mL respectively. Given this 10-fold difference in toxicity it would be expected that a much higher concentration would be needed in *P. mirabilis* relative to that of *P. vulgaris* to damage the pathways involved in swarming motility. The metabolic target of BDM-I in these pathways may be more susceptible or more significant in *P. vulgaris*.

Pre-exposure to BDM-I followed by surface growth on BDM-I-free medium showed that BDM-I could irreversibly damage some part of the pathways involved in

swarming motility for both species. This permanent affect on a metabolic target is consistent with its bactericidal action on *Proteus* spp. (Chapter 2).

A well-known swarming inhibitor, ρ-nitrophenylglycerol (PNPG, is structurally similar to diketopiperazines, natural QS signals that modulate swarming behaviour (Liaw *et al.*, 2000; Liaw *et al.*, 2001). PNPG has been shown to inhibit the expression of virulence factors (urease, haemolysin and protease) and the ability to invade uroepithelial cells but does not show whole cell toxicity (2001). Super-swarming *P. mirabilis* phenotypes, which were partially inhibited by PNPG, showed mutations in the *rsbA* gene encoding a histidine kinase of the two component signalling system. Liaw *et al.* (Grangeasse *et al.*, 2007; Kirstein & Turgay, 2005) hypothesised that RsbA may regulate swarming and virulence factor expression by repression and that PNPG may target RsbA or RsbA-regulated pathways.

Surface swarming is regulated via the flagellar master and QS signalling (Section 4.3.1). Since BDM-I does not affect flagellar motility (data not shown) but does inhibit swarming behaviour then it is probably acting on the signalling pathways involved in QS. Phosphorylation (traditionally on histidine) is a key part of QS in bacteria and tyrosine phosphorylation has found to be involved in similar signalling events (Roberts, 1996). A role for tyrosine phosphatases in QS in swarming motility has not been identified. If PTP's are involved, this would be consistent with the hypothesis that BDM-I may inhibit bacterial protein tyrosine phosphatases (Chapter 5).

Swarming regulation in *Proteus* is associated with the regulation of expression of other virulence factors. Direct effects of BDM-I on exoenzymes in *Proteus* spp. have not been investigated to date.

4.5 CAPSULAR POLYSACCHARIDE PRODUCTION IN *KLEBSIELLA PNEUMONIAE*

4.5.1 Background

Since cell surface polysaccharides are known to play a role in bacterial adherence, resistance to specific and non-specific host immunity and desiccation, all of which contribute to bacterial virulence (Vincent *et al.*, 1999; Vincent *et al.*, 2000; Wugeditsch *et al.*, 2001), it was decided to investigate the effect of BDM-I on a capsule producing strain of *Klebsiella pneumoniae*.

Low molecular weight protein tyrosine phosphatases and their associated protein tyrosine kinases have been shown to be involved in the synthesis and export of capsular polysaccharides in Gram-negative bacteria such as *E. coli* (Grangeasse *et al.*, 1998), *Acinetobacter johnsonii* (Preneta *et al.*, 2002) and *Klebsiella pneumoniae* (Vincent *et al.*, 2000). Most of these genes, which show strong sequence similarity, are located in *cps* operons encoding capsular polysaccharides (CPS), such as K and M antigens and are a conserved feature of Gram-negative bacteria (Stevenson *et al.*, 1996).

CPS production can be regulated positively or negatively by phosphorylation of tyrosine residues. For example, phosphorylation of the PTP Wzc of *E. coli* results in inhibition of the synthesis of colonic acid, a component of the slime polysaccharide or M antigen. Dephosphorylation by the PTP Wzb thus positively regulates the production of colonic acid and inhibition of Wzb results in a decrease in capsule formation in *E. coli* (1991). This finding can be extended to the species which have cognate PTK/PTP enzyme pairs showing gene sequence similarity and which cross-react with *E. coli* Wzc//Wzb. A range of enteric bacteria synthesize colonic acid but it has not been reported in *Klebsiell*a. McCallum & Whitfield (2002) have shown

that the *K. pneumoniae rcsA* gene, involved in expression of K antigen, when cloned into *E. coli* causes a mucoid phenotype resulting from the synthesis of colonic acid. Preneta *et al.* (Bender *et al.*, 2003) reported that the PTK (Yco6) in *Klebsiella pneumoniae* and a PTP (Yor5) are involved in the regulation of CPS (K antigen) synthesis but how this regulation is achieved is not yet fully described.

Tyrosine phosphorylation regulatory systems also play a role in exopolysaccharide production in Gram-positive bacteria. In *Streptococcus pneumoniae*, a PTP (Cps2B) and a PTK (Cps2C/2D) regulate production of Type II CPS. Phosphorylation is essential for CPS production with Cps2C/2D being positive regulators of polysaccharide chain length and similar 'polysaccharide co-polymerases' occurring in Gram-negative bacteria (Domenico *et al.*, 1985). The PTP CpsB is manganese-dependent and does not have sequence similarity to *E.coli* Wzb and related PTP's.

Klebsiella pneumoniae is a significant opportunistic human pathogen causing pneumonia, urinary tract and systemic infections and strains are often multi-drug resistant. The characteristic polysaccharide layer, or capsule, of *Klebsiella pneumoniae* is an important virulence factor and capsule size has been related to pathogenicity (Kadurugamuwa *et al.*, 1985). CPS production is reduced in the presence of cephalosporins, cell wall synthesis inhibitors (2000).

In subcultures from MMC assays, no inhibition of colony mucoidy was observed in *K. pneumoniae* treated with BDM-I in MHB. The ability of BDM-I to interfere positively or negatively with capsule formation by individual cells on direct exposure to BDM-I was investigated by microscopic examination of stained cells.

4.5.2 Materials and Methods

An overnight culture of *K. pneumoniae* in MHB (5 mL) was diluted 1:100 in 50 mL MHB and incubated at 37°C, 160 rpm, 1-2 h until an opacity equivalent to McF 0.5 standard (~1.5×10^6 CFU/mL) was achieved. The culture was divided into aliquots of 10 mL to which were added BDM-I in DMSO at 100× the test concentrations of 32, 128 and 512 µg/mL. Cultures were treated with BDM-I for 8 h. The vehicle control culture contained 1% DMSO. Viable counts were obtained from 10-fold dilutions of cultures on NA incubated for 24 h at 37°C. For examination of capsules, a 5 µL aliquot of cell culture was stained by mixing with 5 µl 5% Congo red on a microscope slide. When the suspension was dry, Manevals' background stain and fixative was applied for 1 min before rinsing in distilled water and examining under the light microscope. The experiment was repeated twice and three slides prepared for each treatment on each occasion. Photographs of stained cells were obtained using oil immersion at 1000× and recorded using a Motic digital camera (Motic China Group, Xiamen, China).

4.5.3 Results

No significant differences in capsule size were observed on microscopic examination of stained cells after exposure for 8 h to 32, 128 and 512 µg/mL BDM-I when compared to an untreated control (Figure 4-7). An assay more directly measuring components of exopolysaccharide secretion might indicate an effect but this was not attempted here given the lack of any obvious effect on visual microscopic examination or examination of cultures.

4.5.4 Discussion

No reduction in capsule size was demonstrated after exposure of *Klebsiella pneumoniae* to BDM-I 512 µg/mL, the highest concentration testable because of insolubility of BDM-I. The geometric mean MIC for *Serratia marcescens* is 800 µg/mL, therefore 512 µg/mL may have been too low a concentration to inhibit exopolysaccharide production.

K. pneumoniae possesses the Yco6 (PTK)/Yor5 (PTP) enzyme pair which is involved in CPS (K antigen) synthesis which have sequence homology with PTK/PTP enzymes involved in exopolysaccharide biosynthesis in *E. coli* (Wzb/Wzc) and *A. johnsonii* (Ptk/Ptp) (3.1.4). A mechanism for PTP action has only been identified in *E. coli* to date. Phosphorylation of the PTK (Wzc) of *E. coli* results in inhibition of the synthesis of colonic acid (M antigen). Dephosphorylation by the PTP Wzb thus positively regulates the production of colonic acid and inhibition of the PTP results in a decrease in capsule formation in *E.coli*. Production of colonic acid (M antigen) has not been demonstrated in *Klebsiella* and it is not known whether Yor5 regulates K antigen production positively or negatively.

If BDM-I is functioning as a tyrosine phosphatase inhibitor, an affect, positive or negative on capsule production might be expected in *Klebsiella*. If tyrosine phosphorylation is associated with increased CPS production, a PTP inhibitor would be expected to decrease prodigiosin production and vice versa. In this preliminary investigation, BDM-I appears not to affect CPS production in *K. pneumoniae*. Further investigation for any effect of BDM-I of the production of colonic acid in *E. coli,* is proposed.

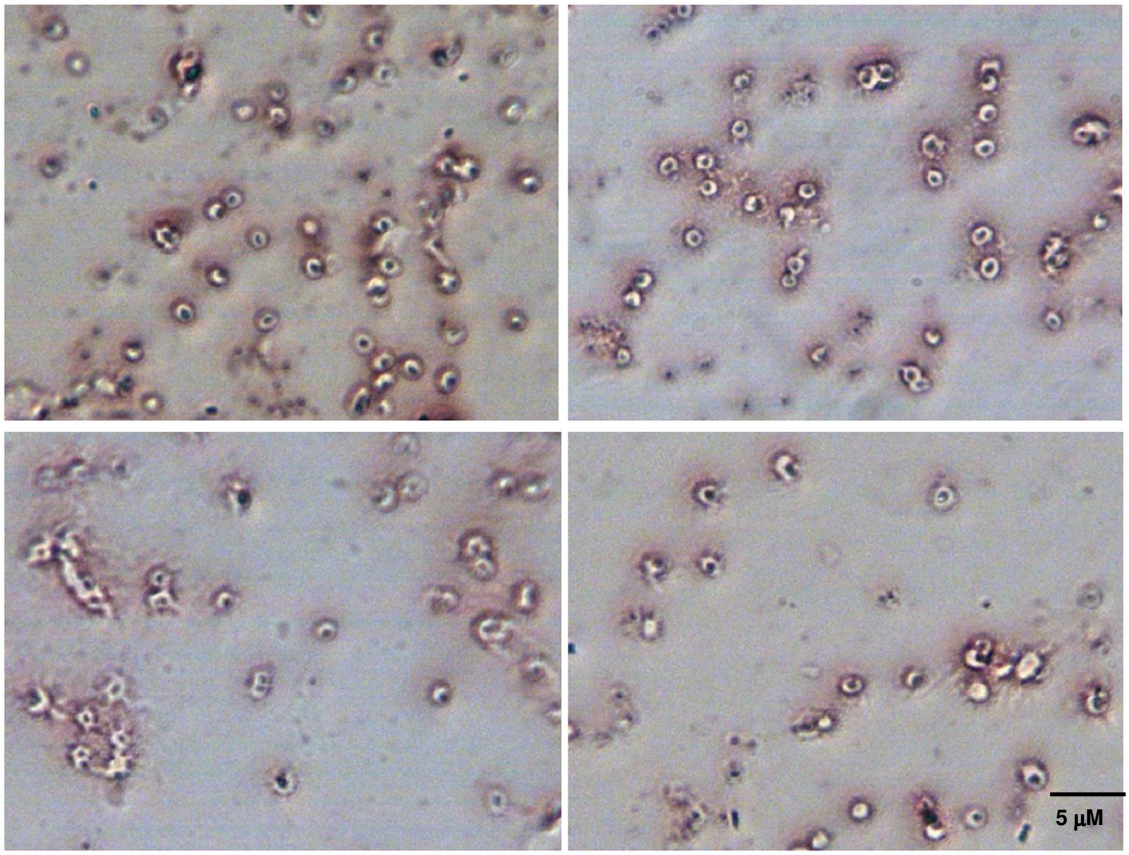


Figure 4-7. *Klebsiella pneumoniae* negatively stained capsules after exposure to BDM-I.

BDM-I exposure was for 8 h. Treatment clockwise from top left: DMSO control, 32 µg/mL, 128 µg/mL, 512 µg/mL. Cells stained with Congo Red and Maneval's Stain and photgraphed using an oil immersion lens at 1000× magnification.

Vincent *et al.* (Galperin & Koonin, 1999) suggest that protein tyrosine phosphorylation is part of a cascade of reactions determining bacterial pathogenicity. BDM-I has been shown to affect swarming motility in *Proteus* and the secondary metabolite prodigiosin but not capsular polysaccharide production in *Klebsiella.* In bacteria, PTP's are more phylogenetically distant and heterogeneous in both distribution and function than in eukaryotes, so differences in inhibitory effects and activities between inhibitors is not surprising.

Inhibition of virulence factors, such as capsule production and swarming motility, in addition to inhibition of growth, is a positive attribute for anti-infective drugs. Their inhibition can play a major role in the prevention of infections and virulence factors have recently been proposed as suitable selective targets for anti-infective drug development (Lambert, 1998).

CHAPTER 5 THE EFFECT OF BDM-I ON CELL FUNCTIONS

5.1 INTRODUCTION

Investigations reported in Chapter 3 have shown that BDM-I does not affect the structural integrity of the bacterial or yeast cell wall and cytoplasmic membrane. BDM-I, as a small lipophilic uncharged molecule would diffuse through lipid membranes. It has been shown to penetrate cells and to act on intracellular pathogens (Chapter 2). Its broad spectrum of activity suggests a major target that is highly conserved across prokaryotic and eukaryotic microorganisms. In Chapter 4, BDM-I was shown to inhibit metabolic pathways in bacteria involved in survival responses such as swarming motility and secretion of protective pigments. This chapter presents investigations into the effect of BDM-I on three highly conserved metabolic functions: bacterial enzyme systems involved in ATP synthesis, RNA transcription and translation and intracellular signalling via protein tyrosine phosphatases.

Respiration and protein synthesis are cellular functions which are universal and sufficiently diverse across cell types to provide selective targets for clinically effective anti-infective drugs (Shawver *et al.*, 2002). Protein kinases and phosphatases that coordinate the regulation of cellular responses to multiple environmental signals are also sufficiently diverse to provide selective targets (Section 1.3.3). Drugs inhibiting the tyrosine kinase activity of epidermal growth factor receptor and vascular endothelial growth factor receptor are in clinical use or clinical development for the treatment of cancer (Lee & Wang, 2007). Protein tyrosine phosphatases specifically, have emerged recently as promising drug targets with growing evidence of the importance of PTP1B in human conditions such as diabetes and obesity (Madigan *et al.*, 2006). No drugs targeting the kinases or phosphatases of microbial pathogens are reported to be in development to date.

5.2 ATP PRODUCTION BY ATP BIOLUMINESCENCE ASSAY

5.2.1 Background

The cell membrane of prokaryotes is more functionally active than that of eukaryotes. Many enzyme systems that occur in organelles in eukaryotes are located in the cell membrane in bacteria, such as those for ATP synthesis and oxidative phosphorylation, where they are readily accessible to antimicrobial compounds. ATPase, or ATP synthase, is a multi-component enzyme which catalyses the synthesis of ATP using the electrochemical ion gradient (H^+ or sometimes Na^+) generated by oxidative phosphorylation (Deckers-Hebestreit & Altendorf, 1996). ATP synthase is fully reversible and can hydrolyse ATP to generate a proton gradient. It consists of the F_0 component embedded in the membrane and attached by a stalk to the F_1 component, which projects from the cytoplasmic face of the bacterial plasma membrane (Futai & Kanazawa, 1983). ATP synthase enzymes are highly conserved but do show structural variations. The bacterial F_1F_0ATPase (F type) is essentially the same in structure and function as the F_1F_0ATPases from mitochondria and chloroplasts of eukaryotic cells but differs from the ATPase's in fungal, plant and animal cell membranes (Kuyyakanond & Quesnel, 1992).

Interference with ATPase can be secondary to disruption of membrane integrity, depolarisation of the membrane or a direct result of action on the enzyme also causing depolarisation. Most ATP synthesis inhibitors are biocides, such as phenolic compounds and chlorhexidine and inhibition is secondary to membrane disruption or depolarisation (Dinning *et al.*, 1998). Pyrithiones (1-hydroxy-2-pyridinethiol sodium salt and zinc chelate) depolarise cell membranes and markedly reduce intracellular ATP levels in *E. coli* and *Pseudomonas aeruginosa* washed cells within 1 h of exposure to sub-inhibitory concentrations (Perlin *et al.*, 1985). Despite the similarity among the

F_1F_0ATP synthases, selective inhibitors have been reported. Oligomycin only weakly inhibits bacterial ATP synthase but strongly inhibits mitochondrial ATP synthase while venturicidin strongly inhibits bacterial ATPase (Andries *et al.*, 2005). A diarylquinoline was identified from resistant mutant studies, as a probable inhibitor of *atpE*, whose product is the proton pump of the ATP synthase of mycobacteria (Denisenko *et al.*, 27 Dec. 2002 International patent). This is a very selective target and the high bactericidal activity was shown to be highly specific to mycobacteria. Diarylquinolines are in further development as drugs for the treatment of mycobacterial infections. BDM-I has a high level of activity against *Mycobacterium tuberculosis* with an MIC of ~6 µg/mL (Deckers-Hebestreit & Altendorf, 1996; Rao *et al.*, 2001).

Although BDM-I does not disrupt the cell membrane, it could directly affect membrane-bound enzyme systems such as ATPases in bacterial cells and mitochondria or the enzymes involved in the electron transport chain. Inhibition of ATP synthase leads to depletion of intracellular ATP and imbalance in pH homeostasis (Hattori *et al.*, 2003; Stanley, 1986).

The effect of BDM-I on ATP production in *Bacillus cereus* and *Staphylococcus aureus* is investigated using a bioluminescent assay. BDM-I is bactericidal to *B. cereus* and bacteriostatic to *S. aureus*. Both use oxidative phosphorylation for synthesis of ATP when growing aerobically. ATP production is an indicator of metabolically active cells as it is positively correlates with cell numbers in actively metabolising cells.

5.2.2 Materials and Methods

The Promega Bac-Titre™ Glo assay was used to determine whether inhibitory concentrations of BDM-I have a direct effect on ATP production in bacteria. Briefly, ATP was extracted at intervals from log phase cells growing aerobically and

ATP production measured by the luminescent signal generated by a luciferase detection system. Beetle luciferin in the presence of ATP is oxidized to a luminescent oxyluciferin by a recombinant firefly luciferase and is detected spectrophotometrically. The assay has been validated for *Bacillus cereus* and *Staphylococcus aureus*.

The Bactitre-Glo™ reagent, containing a cell ATP extraction system and a luciferase-based detection system, was prepared according to the manufacturers instructions (Technical Bulletin 337, revised 04/04, Promega, WI, USA) and stored at -80°C in 1 mL, single use aliquots. Each aliquot was thawed, brought to room temperature and protected from degradation by light before use.

For each assay, *Bacillus cereus* and *Staphylococcus aureus* were grown overnight in 5 mL MHB and diluted to ~1×10^6 in MHB. BDM-I was added from 100× stock solutions to give a final concentrations of 16 and 32 µg/mL for *B. cereus* and 8 and 32 µg/mL for *S. aureus* with 1% v/v DMSO. Treated and untreated control cultures were incubated aerobically for 4 h at 37°C on a shaker (Ratek) at 160 rpm. At 0, 1, 2 and 4 h, 100 µL was removed from each culture for luminescence measurements and viable cell counts. For luminescence, Bactitre-Glo™ reagent (100 µL) was added, the suspension vortexed and incubated for 5 min in the dark at room temperature. Luminescence in each tube (triplicate samples) was measured in a Luminometer (TD-20/20, Turner Designs, Ca, USA) and recorded as Luminometer Units (LU). For viable counts, the 100 µL aliquot was diluted to 10^{-2}, plated onto NA and incubated aerobically at 37°C for 24 h. Assays were completed at least twice on separate occasions. Viable counts were reported as CFU/mL and plotted against time.

An ATP standard curve was produced using adenosine 5-triphosphate standard disodium salt trihydrate (FLAAS, Sigma-Aldrich) at 10-fold concentrations (nM) in MHB instead of culture and proceeding as above. Using the standard curve,

Luminescence Units were converted to units of ATP (1 nM = LU × 0.016 + 4.34) and plotted against time. Correlation of ATP synthesis with cell numbers (viable count) was tested by regression analysis (Excel Data analysis, Microsoft®, 2003).

5.2.3 Results

A standard curve with adenosine 5-triphosphate disodium salt trihydrate showed linearity over a range of 10-fold concentrations from 0.1 to 100 nM ATP (Figure 5-1).

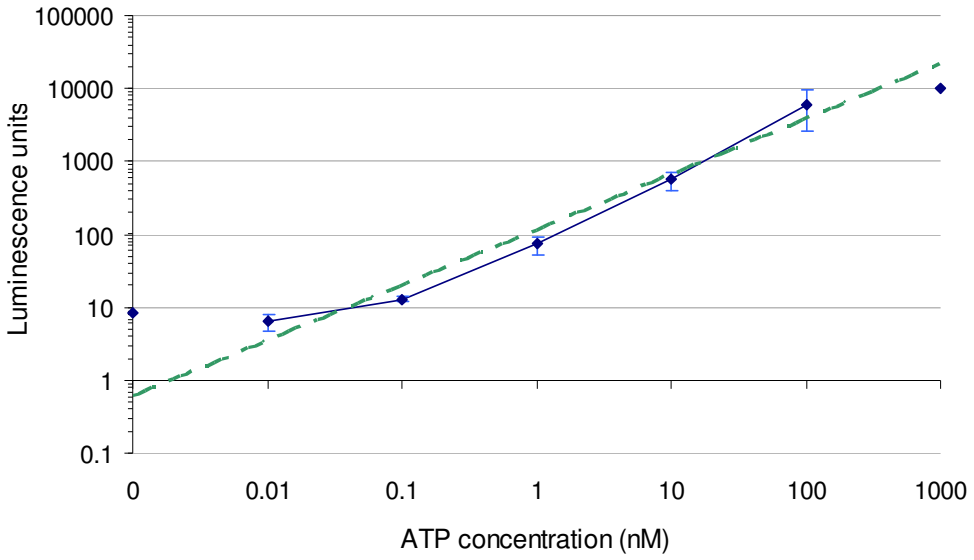


Figure 5-1. ATP Standard curve generated for the BacTitre-Glo™ ATP assay.

Error bars represent standard deviation. Linear trendline is shown dashed.

Maximum measurable luminescence for *B. cereus* untreated control was reached at 4 h and for *S. aureus* at 2 h. Production of ATP in *B. cereus* and *S. aureus* and exposed to BDM-I at inhibitory concentrations of 16 and 32 µg/mL compared to untreated cultures is shown in Figure 5-2. Regression analysis of the curves is shown in Table 5-1. ATP production shows good correlation of ATP production with increase in cell numbers in all treatments for *B. cereus*. Correlation of ATP production with cell

numbers was not as good for *S. aureus* which grew faster than *B. cereus* under these experimental conditions. BDM-I is bacteriostatic for *S. aureus* and at 32 µg/mL (8× the MIC) cell numbers increased ~5-fold over 4 h while ATP production increased ~3.3-fold. The poorer correlation for the untreated control may be due to reaching maximum measurable luminescence at 4 h. The results suggest that BDM-I has not inhibited ATP production more than would be accounted for by a drop in the number of metabolising cells.

Table 5-1. Regression analysis of ATP production and CFU/mL for each treatment.

	B. cereus			*S. aureus*		
	Control	16 µg/mL	32 µg/mL	Control	8 µg/mL	32 µg/mL
R^2	0.94	0.75	0.91	0.79	0.74	0.67
F statistic	30.18	6.05	21.37	7.33	5.68	4.13
p value	0.03	0.13	0.04	0.11	0.14	0.18

5.2.4 Discussion

Measurement of ATP was used here to investigate the effects of BDM-I on total ATP production. The assay detects intracellular and extracellular ATP. However, cells in this experiment are growing exponentially in optimal conditions and nearly all cells would be viable and producing ATP by oxidative phosphorylation. Therefore the ATP measured would be predominantly intracellular ATP generated by membrane-bound ATP synthase. Inhibition of ATPase can suggest disruption of membrane integrity or depolarisation of the membrane directly or by action on the enzyme.

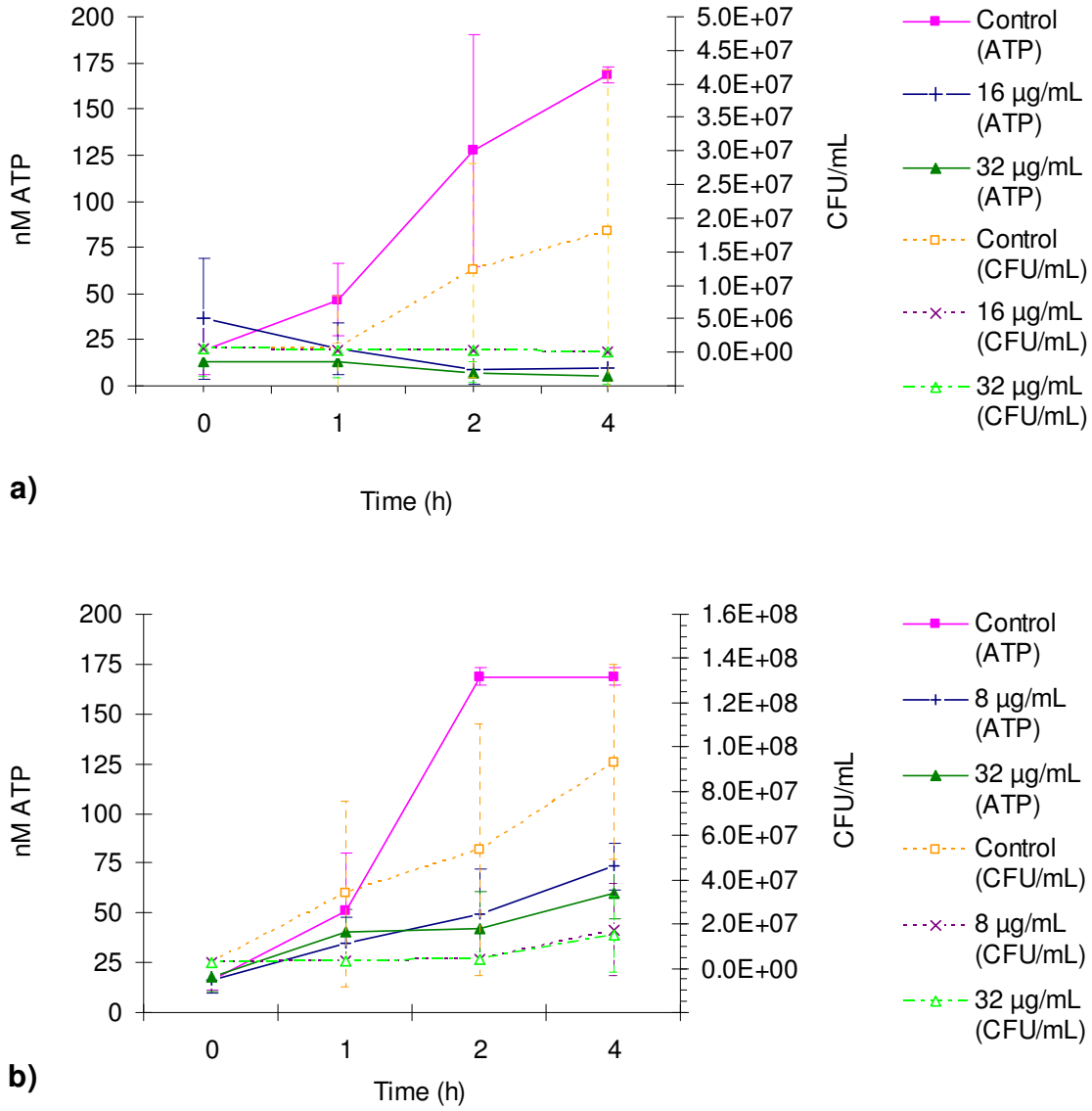


Figure 5-2. Effect on total ATP production (nM) by *B. cereus* (a) and *S. aureus* (b) in MHB exposed to BDM-I (µg/mL).

ATP (solid line) and viable cell numbers (dotted lines) were measured at each time point by luminescent assay and viable counts respectively as described in Section 5.2.2. Ctrl, MHB with bacteria without BDM-I. Each line represents a different concentration of BDM-I (µg/mL).

The results suggest that BDM-I does not directly inhibit ATP production and would indicate that it does not adversely affect the enzyme systems in the cell membrane involved in aerobic ATP production, either ATPase or the enzymes of the electron transport chain. This is consistent with observation of an equal inhibitory effect by BDM-I on *Bacillus, Staphylococcus* and yeast spp. whether cells were grown in aerobic conditions or forced into fermentative energy production under anaerobic conditions (Nicoletti *et al*., unpublished).

Cell types vary considerably in ATP content and intracellular concentrations are affected by a number of factors (Artsimovitch & Vassylyev, 2006). *S. aureus* grew faster than *B. cereus* in this assay, reaching the maximum measurable luminescence (10,000 LU) at 2 h compared to 4 h for *B. cereus*. It is also able to grow slowly in the presence of 512 µg/mL BDM-I (256× MIC). Therefore the use of much higher concentrations where an inhibitory effect would be more obvious might demonstrate some direct interference with ATP production.

5.3 TRANSCRIPTION/TRANSLATION ASSAY USING CELL-FREE *E. COLI* SYSTEM

5.3.1 Background

Inhibition of RNA transcription and inhibition of translation result in inhibition of protein synthesis and are important targets for antimicrobial agents. Aminoglycosides, tetracyclines, macrolides, lincosamides and chloramphenicol are major antibiotic classes inhibiting the translation of mRNA. Bacterial DNA-dependent RNA polymerase for transcription of mRNA is a less well exploited target. The rifamycins (eg. rifampin) inhibit RNA polymerase (RNAP) and are the only class of inhibitors used clinically (2004).

Cell-free extracts which allow transcription and translation of mRNA are widely-used to investigate protein synthesis *in vitro*. One of the most frequently used assay tools is a cell-free *E. coli* transcription/translation system which can be applied to the screening of compounds that may affect transcription or translation of a DNA template. BDM-I was screened in such a cell-free assay system for inhibition of the transcription of the β-galactosidase gene and the synthesis of the enzyme.

5.3.2 Materials and Methods

The Promega '*E. coli* S30 Extract for Circular DNA' contains all the macromolecular components required for transcription of an exogenous DNA template (generation of mRNA, tRNA and rRNA) and translation (synthesis of protein) and the necessary amino acids, energy sources, energy generating systems and cofactors for protein synthesis. A control DNA template (pGEM® β-Gal) containing the coding sequence of β-galactosidase downstream of an *E. coli* wild type lacZ promoter, allows direct measurement of synthesised β-galactosidase from the conversion of ONPG (o-nitrophenyl-β-D-galactosidase, colourless) to ONP (o-nitrophenyl, yellow). ONP production, measured spectrophotometrically, is proportional to the amount of protein synthesised.

The assay was performed based on the method of Ulvatne *et al.* (Ishihama, 2000). Doubling dilutions of BDM-I (8 – 128 μg/mL), were prepared in 60% DMSO so that the final percentage did not exceed the recommended maximum of 5%. A 96-well white LIA microplate (Lumitrac® 200, Greiner Bio-one, Germany) was prepared with BDM-I (15 μL). A vehicle control well received 15 μL of 60% DMSO vehicle. The reaction control for maximum protein synthesis (no BDM-I) and negative control (no extract and no BDM-I) wells received nuclease free water. Amino acids (2 μL) and S30 premix (8 μL) (Promega, WI, USA) were thawed and added to all wells on the plate. *E.*

coli S30 extract (5 µL) was added to all treatment wells and the reaction control. The negative control received nuclease free water. Nuclease-free water (20 µL, Promega) was added to wells to an assay volume of 50 µL and the plate incubated at 37°C for 30 min. DNA (1 µL of 0.25 mg/mL pGEM® β-Gal, Promega) was added to all wells and the plate re-incubated at 37°C for 30 min. After incubation, 150 µL of 15 mM ONPG (Sigma-Aldrich) was added and OD_{420nm} recorded every 10 min for 2 h to measure the amount of ONP produced. An excess of ONPG is used to ensure that the rate limiting step in the conversion of ONPG to ONP is the amount of enzyme. Some precipitation of BDM-I was noted at 128 µg/mL. Since the amount of enzyme is directly proportional to ribosomal activity, the amount of ONP generated can be used as a measure of protein synthesis.

Assays were replicated three times with measurements in triplicate. The mean OD_{420nm} was plotted against time. Slopes of the curves (rate of production of ONP) were compared visually. Relative rates of protein synthesis (corrected for the slope of the negative control) were calculated if necessary as slope of curve with BDM-I/slope of positive reaction curve (no BDM-I).

5.3.3 Results

There was no change in the rate of protein synthesis (as measured by the rate of hydrolysis of ONPG to ONP in the presence of increasing concentrations of BDM-I up to 16× higher than the MIC (Figure 5-3). Slopes were comparable on visual inspection and relative rates of protein synthesis were therefore not calculated. BDM-I did not inhibit the transcription or translation of β-galactosidase. BDM-I had no effect on ribosomal functions and/or on bacterial DNA-dependent RNA polymerase *in vitro*.

There was no interference with absorbance readings from BDM-I in the absence of pGEM-βgal DNA (data not shown) as these values were similar to the negative control which lacked the S30 extract template. The higher absorbance values recorded for higher concentrations of BDM-I (64 and 128 µg/mL) are possibly due to the insolubility noted at 128 µg/mL and/or interference at the 420 nm wavelength since BDM-I has maximum absorbance at 370 nm.

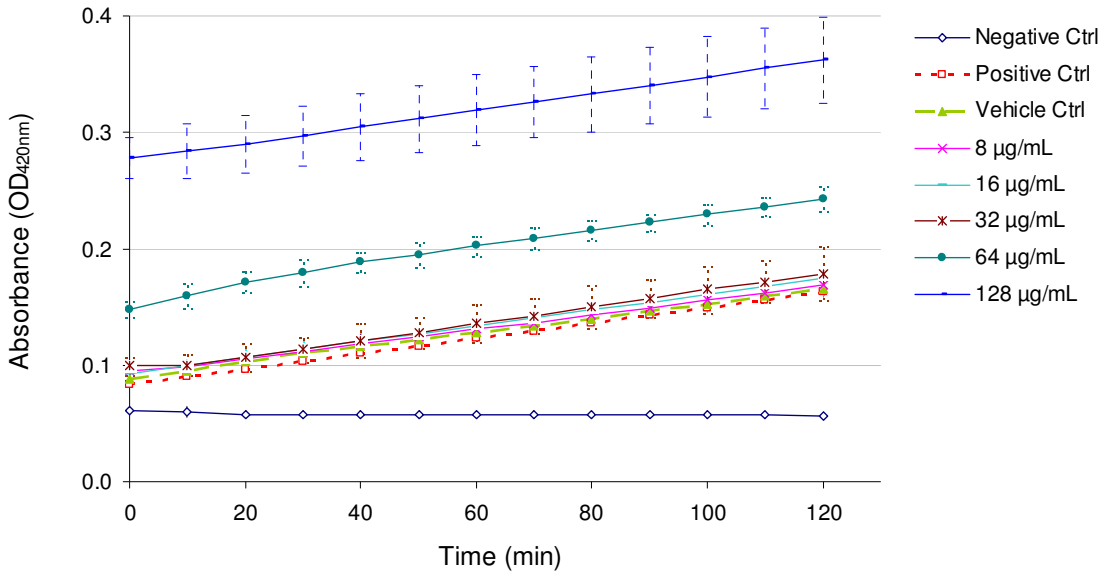


Figure 5-3. Effect of BDM-I (µg/mL) on synthesis of β-galactosidase by measurement of ONP (OD_{420nm}).

Cell-free protein synthesis assay using *E. coli* S30 extract (Promega) with pGEM® β-Gal (Promega) as the DNA template. Error bars represent standard deviation.

5.3.4 Discussion

The lack of effect of BDM-I on cell-free protein synthesis seen here supports the observation of a lack of interaction (synergistic or antagonistic) with antibiotics affecting different stages of protein synthesis (Section 2.7).

Genes coding for proteins are transcribed by multi-unit DNA-dependent RNA polymerases. Eukaryotes, have three distinct RNA polymerases (I, II and III), each responsible for the transcription of a different class of RNA. In bacteria, transcription is accomplished by RNA polymerase (RNAP) with a conserved core linked to a sigma subunit that specifies binding to promoter elements and transcript regulators. In *E. coli*, more than 100 additonal factors have been identified which modify the activity of RNAP (Iyer *et al.*, 2004). Unlike in Archea and Eukarya, bacterial RNAPs show considerable variation in RNAP domains across prokaryotic lineages (Murray *et al.*, 2001; Pratt *et al.*, 2004). Extracted enzyme systems from one species, therefore, may not be as sensitive in detecting inhibition by an antimicrobial agent of the RNAP of another species. Reports using assays based on extracted enzyme systems suggest variable responses to inhibitors by *E. coli*, *S. aureus* and *Streptococcus pneumoniae* (Tonks, 2006; Zhang, 2002). This limits the extrapolation of data to other species. While indicative, the failure of BDM-I to inhibit transcription or translation in *E. coli* should be prudently extended to other bacterial and eukaryotic pathogens.

5.4 PROTEIN TYROSINE PHOSPHATASE ENZYME ASSAY

5.4.1 Background

The role of tyrosine phosphatases in eukaryotic and prokaryotic cells has been discussed briefly in Chapter 1 (Section 1.3.3). Tyrosine phosphorylation in eukaryotes, controls many important cell functions: cell growth and differentiation, the cell cycle, cell-to-cell communication, cell migration, gene transcription, apoptosis and the immune response (Kennelly, 2002). Tyrosine phosphorylation is less common in bacteria and less well investigated. Conventional PTP, Dual specificity phosphatases (DSP) and low molecular weight phosphatases (LMW-PTP's) have been identified in bacteria (Grangeasse *et al.*, 2007). They regulate particular functions in bacteria which

are different from those in eukaryotes. These include stress responses and virulence mechanisms such as secretion of exopolysaccharides and proteins and production of secondary metabolites (Tonks, 2006).

All PTP families share a common and highly conserved catalytic site which binds phosphate residues on tyrosine. Conventional PTP's contain a catalytic site of approximately 280 amino acids which contains an invariant signature motif ($(H)CX_5R$) or "PTP loop" (Guan & Dixon, 1991). Residues in this motif form a phosphate binding loop in which a cysteine and an arginine separated by any five amino acid residues. The cysteine residue acts as a nucleophile and is essential for phosphate binding, the arginine residue is critical for substrate binding and stabilisation of the enzyme-substrate intermediate and an aspartate residue acts as acid or base during catalysis (Guan & Dixon, 1991; Zhang *et al.*, 1994a; Zhang *et al.*, 1994b). The thiol anion of the cysteine residue initiates the reaction through nucleophilic attack on phosphate to form the intermediate, coupled with protonation of tyrosine on the substrate by the adjacent aspartic acid residue. Tonks (2002) and Zhang (Park & Pei, 2004) have reviewed the structure, function and substrate specificity of PTP's.

The species-specific effects noted for BDM-I on swarming motility in *Proteus* and on prodigiosin production in *Serratia* suggested that one MOA might be interference with aspects of QS involved in cell-to-cell communication and the export of cell products. That insight led to a review of the literature on cell signalling and its regulation by phosphorylation in bacteria.

Benzyl nitroalkenes related to BDM-I have been shown to be slow, reversible inhibitors of the tyrosine phosphatases PTP1B and Yop (2004). They showed that benzyl-β-nitroethene acts as a tyrosine mimetic with the nitroethene chain pharmacophore forming a reversible, time-dependent covalent complex with cysteine at

the catalytic site. Park & Pei (Park & Pei, 2004) also showed that the position of cysteine at its position in the catalytic loop was critical to inhibition by benzyl nitroethene and that a nucleophilic attack on the nitro group by the thiolate anion of cysteine formed a reversible covalent adduct. They further showed that there was no nucleophilic addition at the alkene double bond as happens with the interaction with thiols (Section 2.4). The double bond is not attacked by cysteine and appears necessary for phosphatase inhibition through the maintenance of a rigid planar geometry, critical for positioning the nitro group and/or for binding (Zhang, 2002).

The conserved nature of PTP's across prokaryotic and eukaryotic cells and the phylogenetic differences in structure and function makes them a plausible target for a compound with a broad spectrum of activity such as BDM-I. The hypothesis that BDM-I, a tyrosine mimetic (Figure 5-4), acts as an inhibitor of bacterial PTP's was proposed. The ability of BDM-I to inhibit human and bacterial tyrosine phosphatases was therefore tested by enzymic assay. The hypothesis was also reviewed for compatibility with the results of all investigations into the activity of BDM-I on bacterial and yeast cells undertaken to date.

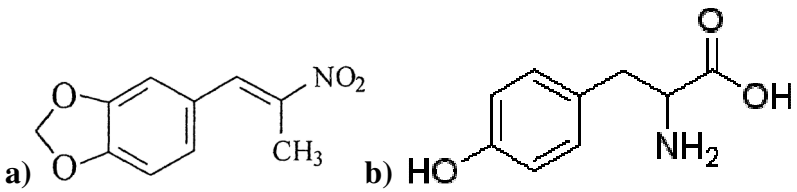


Figure 5-4. Comparison of structures of a) BDM-I and b) Tyrosine

Assayed phosphatases

PTP1B, classified as a conventional PTP, is the most well-studied of the PTP's as it was one of the first characterised in humans. It plays a role in cellular signalling defects relating to cancer, diabetes and obesity. PTP1B is a negative

regulator of the insulin-stimulated signal transduction pathway, binds to the epidermal growth factor receptor and is over-expressed in many cancers and is a target for inhibitor development (Taing *et al.*, 1999). It possesses the characteristic catalytic phosphate binding site and a second, adjacent, non-catalytic binding site, also for aryl phosphates (Pacitti *et al.*, 1994). The latter is not as highly conserved and is suggested as a target for selective PTP1B inhibitors. PTP1B is an intracellular enzyme with high activity, making it especially useful for screening inhibitors of phosphorylation reactions, phosphorylation kinetics and substrate specificity.

Human CD45 is a transmembrane dual-specificity phosphatase (DSP), also known as leukocyte common antigen (LAC) (Pacitti *et al.*, 1994). DSP's can dephosphorylate a tyrosine and either a serine or threonine residue on the same protein. CD45, a glycoprotein expressed on immune cells, dephosphorylates both tyrosine and serine and plays a crucial role in regulating signal transduction leading to T-cell mediated immune responses (Kennelly, 2002; Zhang, 2002). CD45 is routinely used for regulation and inhibition studies in the immunological field. No ortholog of CD45 has been discovered in bacteria (Bliska *et al.*, 1991; Viboud & Bliska, 2005).

Yop is one of the first bacteria-derived PTP's available in a purified form. Yop proteins have sequence and structural similarity to eukaryotic conventional PTP's and are encoded in a virulence plasmid of pathogenic *Yersinia* species which also encodes a Type III secretion system (TTSS). *Yersinia* export these toxic effector proteins (Yop's) through TTSS into host cells where they promote virulence through host protein dephosphorylation and interference with host signal transduction involved in adhesion, phagocytosis and killing of bacteria (Yao *et al.*, 1999). It is an outer membrane protein of *Yersinia* spp. that is active in host cells, its phosphatase activity altering host cell function and immune responses to benefit the bacterium (2004).

An enzyme assay was used to investigate the ability of BDM-I to inhibit PTP1B, Yop and CD45. A fluorescent assay was chosen for its sensitivity and compatibility with these enzymes.

5.4.2 Materials and Methods

Assay principle

Phosphatase activity was investigated using the ProFluor™ Tyrosine Phosphatase Assay, V1281 (Promega). The selected PTP's are able to dephosphorylate a Rhodamine 110 substrate (bis-amide rhodamine 110 phosphopeptide). In the bis-amide form, R110 fluorescence is suppressed. Dephosphorylation of this substrate enables cleavage by protease and release of highly fluorescent R110. Therefore, the intensity of fluorescence is directly proportional to the activity of the phosphatase. Conversely, inhibition of phosphatase activity will result in the persistence of the phosphorylated substrate and greatly decreased fluorescence. The positive control, vanadate, inhibits both PTP's and DSPs. A fluorogenic peptide, alanine-alanine-phenylalanine linked to 7-amino-4-methyl-coumarin (AAF-AMC,) which cannot be phosphorylated or dephosphorylated but is degraded by proteases, is included in the assays to identify protease inhibitors that are not phosphatase inhibitors. Liberation of free AMC is quantified at excitiation and emission wavelengths different from those used to detect R110 fluorescence. If the protease is inhibited and cleavge of AAF-AMC is prevented, a decrease in AMC fluorescence will accompany the change in R110 fluorescence. AMC fluorescence should remain high while R110 fluorescence decreases in presence of phosphatase only inhibitors.

Assays were carried out according to the manufacturer's recommendations (Technical Bulletin 334, revised 01/04, Promega). The phosphatases (PTP1B, 539735; Yop, 539734 and CD45, 217614) were sourced from Calbiochem (Merck, Darmstadt,

Germany). The specific activity of each enzyme was 75 U/mg protein, 100,000 U/mg protein and 148,375 U/mg protein respectively. Enzymes were aliquoted for individual experiments to avoid repetitive freezing and thawing.

Enzyme titration

Each enzyme was titrated with the Promega reagents to determine the amount required to achieve 80% of the maximum fluorescent signal which is considered optimal for screening assays. Reagent master mixes were prepared in appropriate volumes according to the following schedule:

Reagent	Component	µL component /mL solution
Phosphatase dilution solution	Promega 5× reaction buffer B	200
	Sterile MilliQ water	800
Peptide solution	Promega 5× reaction buffer B	200
	Promega R110 substrate	1
	Promega AMC substrate	1
	Sterile MilliQ water	798
Protease solution	Promega termination buffer C	200
	Promega protease reagent	20
	Sodium vanadate, 100 mM	2
	Sterile MilliQ water	778
Stabiliser solution	Promega termination Buffer C	20
	Promega stabiliser reagent	5
	Sodium vanadate, 100 mM	2
	Sterile MilliQ water	973

MilliQ water is ultrapure water derived from a Millipore system

All reagents were kept on ice until 10 min prior to addition to the plate when they were brought to room temperature. Reaction wells were prepared in duplicate and the enzyme titration performed on two separate occasions. Each well of a 96-well white luminescence microplate (Greiner, Lumitrac® 200, 655075), except column 12 (negative enzyme control well), received 25 µL of Phosphatase dilution solution. A 50

µL aliquot of each enzyme was added into each of two wells in separate rows. Enzymes were serially diluted 2-fold on the plate by removing 25 µL into the following 11 wells.

Peptide solution (25 µL) was then added to each well to initiate the reaction. The plate was spun to ensure all reagents were mixed before incubation at 24°C for 60 minutes in the dark. Plates were spun for 1 min at 200 ×g in a benchtop centrifuge (3K15, Sigma Laborzentrifugen). Protease solution, 25 µL, was added to all wells. The plate was spun and incubated for a further 30 minutes. Fluorescence stabiliser solution (25 µL) was added to each well, the plate spun and the fluorescent signals read at two sets of wavelengths (FLUOstar Optima, BMG laboratories). An excitation of 485 nm and emission of 520 nm was used to detect the signal indicative of enzyme activity on the R110 substrate. An excitation of 355 nm and an emission of 460 nm were used to detect the signal indicative of non-specific inhibition (control AMC substrate). Standard curves were generated for each enzyme and enzyme concentrations producing 80% of the maximum signal calculated as optimal for the screening assays.

Screening assays

For screening assays, 5 µL BDM-I (10× test concentration in 10% DMSO/ milliQ water) was added to appropriate wells in duplicate. Sodium orthovanadate (Na_3VO_4, Calbiochem, Merck) was included on the plates (10× test concentration in 10% DMSO/milliQ water) as a positive inhibitor control. Final test concentrations of BDM-I and sodium orthovanadate ranged between 0.5 µM and 100 µM. Control wells received the vehicle only. Half the control wells (negative reaction controls) received 20 µL control buffer. All remaining assay wells, including the enzyme control wells, received 20 µL phosphatase enzyme preparations to give required enzyme

concentrations. The reaction was initiated by the addition of peptide solution and continued as above.

Duplicated fluorescence values were averaged and combined averages from 2-4 individual assays were graphed. Phosphatase activity (% maximum R110 fluorescence) was plotted against $\log_{10}$ BDM-I concentrations (μM) ±standard deviation (SD) for each enzyme.

Inhibition assay data was required to meet a number of arbitrary criteria in order to be deemed valid. In each assay AMC fluorescence values had to be stable and vary ≤25% from the mean; the maximum R110 fluorescence had to be above 10,000 units; the positive enzyme control well in each case had to read ≥70% maximum R110 fluorescence. Only assays that satisfied these criteria for both sodium orthovanadate and BDM-I were included.

5.4.3 Results

The optimal concentration of each enzyme for use in inhibition assays was determined empirically from standard curves (Figure 5-5 and Figure 5-7). The concentration recommended by the manufacturer is that which produces 80% of the maximum fluorescence signal. Optimal concentrations were estimated from $\log_{10}$ scale graphs as shown in Figure 5-5 and Figure 5-7 for each enzyme.

The AMC fluorescent compound is provided as an internal control to test for agents that may inhibit the protease and therefore produce non-specific fluorescence at a different wavelength than the R110 substrate. The AMC substrate was included in each reaction well and the results demonstrated no interference by sodium orthovanadate up to 100 μM. BDM-I did cause a decrease in AMC fluorescence when

present at concentrations over 100 µM therefore these results for R110 fluorescence are not shown.

BDM-I was a considerably less potent inhibitor of tyrosine phosphatases than vanadate and its activity was not as uniform. Concentrations of sodium orthovanadate resulting in 50% inhibition were ≤40 nM for all enzymes. BDM-I showed 50% inhibition at concentrations of 16 and 25 µM for PTP1B and Yop respectively (Figure 5-6a & b). BDM-I was less effective against CD45 producing a maximum inhibition of 36% at 80 µM (Figure 5-8).

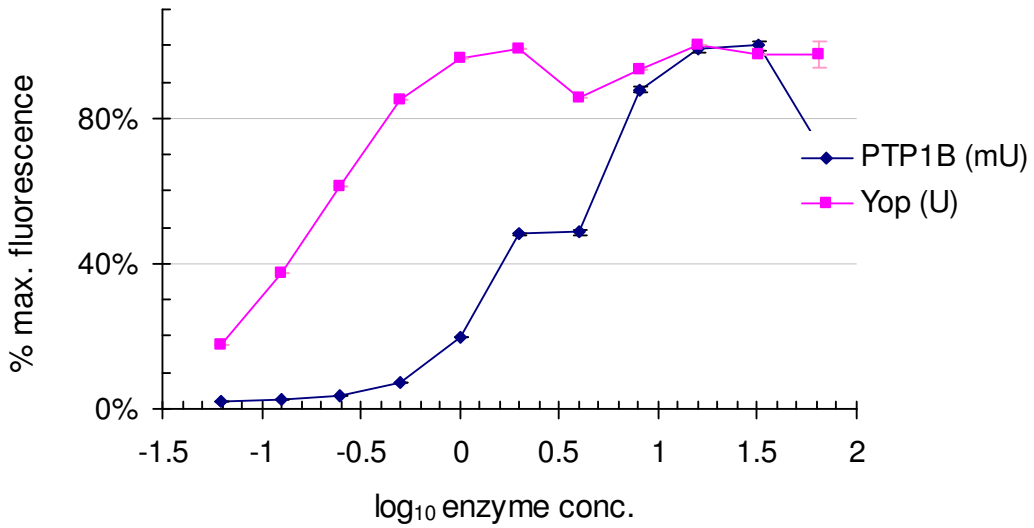


Figure 5-5. Standard curve of PTP enzymes PTP1B and Yop.

Optimum concentration for inhibition assays was estimated as the amount achieving 80% fluorescence. Units of concentration for each enzyme on the x-axis are shown in brackets. Error bars represent standard deviation.

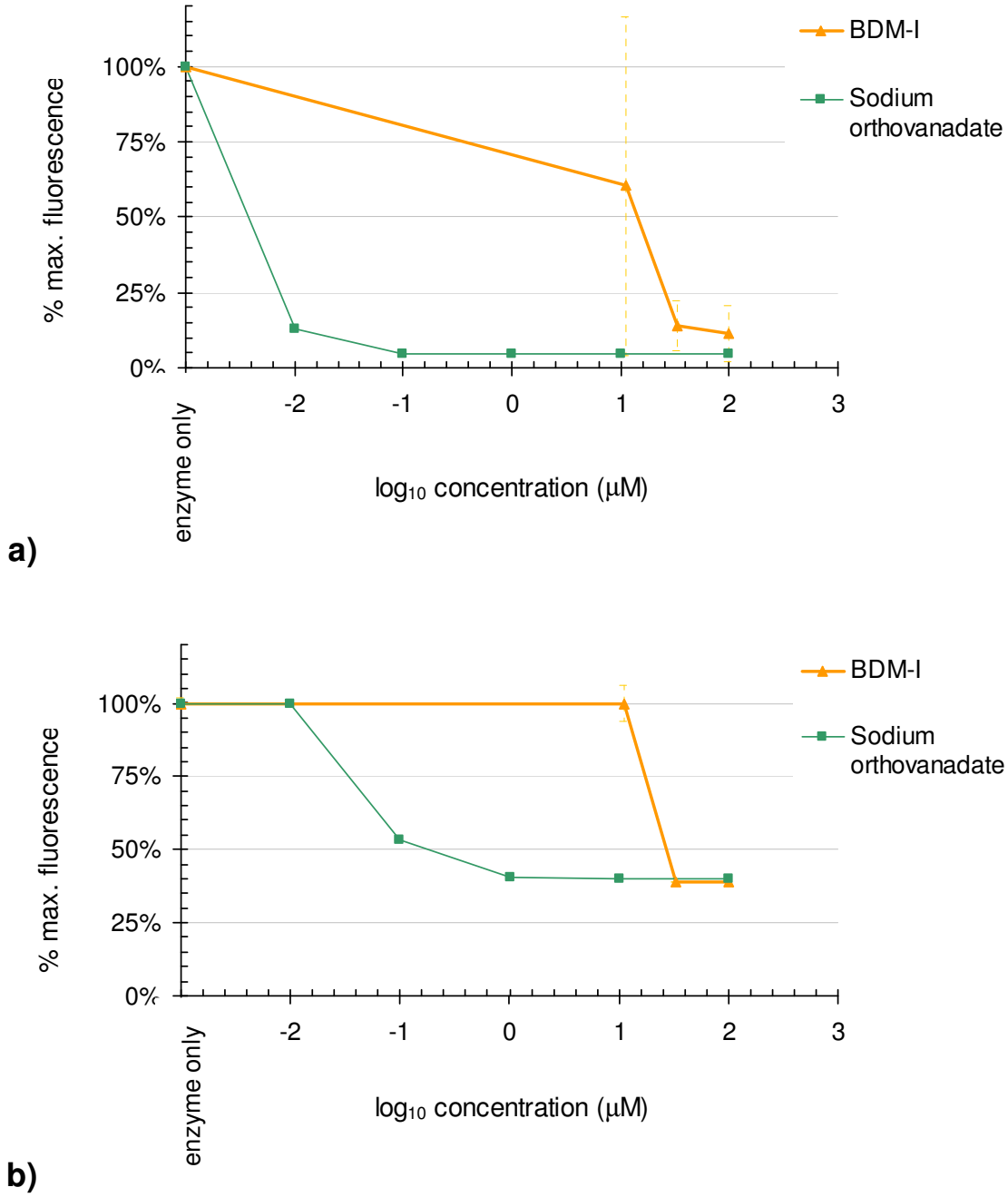


Figure 5-6. Effect of BDM-I and sodium orthovanadate on fluorescence of R110 substrate for PTP enzyme assays using PTP1B (a) and Yop (b).

The amount of PTP1B and Yop enzymes used were 6 mU and 0.6 U respectively. Error bars represent standard deviation.

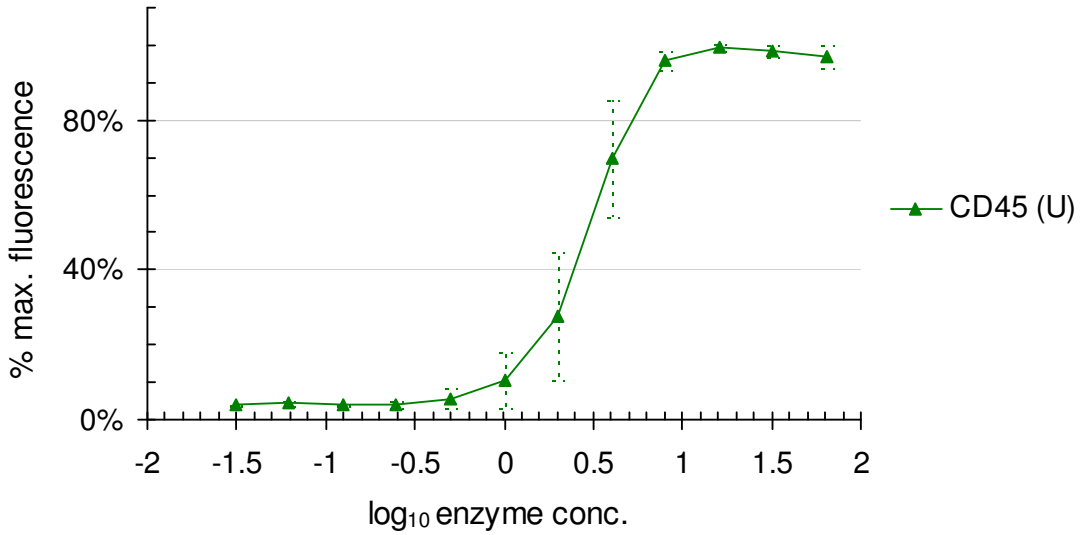


Figure 5-7. Standard curve of PTP enzyme CD45.

Optimum concentration for inhibition assays was estimated as the amount achieving 80% fluorescence. Units of concentration on the x-axis are shown in brackets. Error bars represent standard deviation.

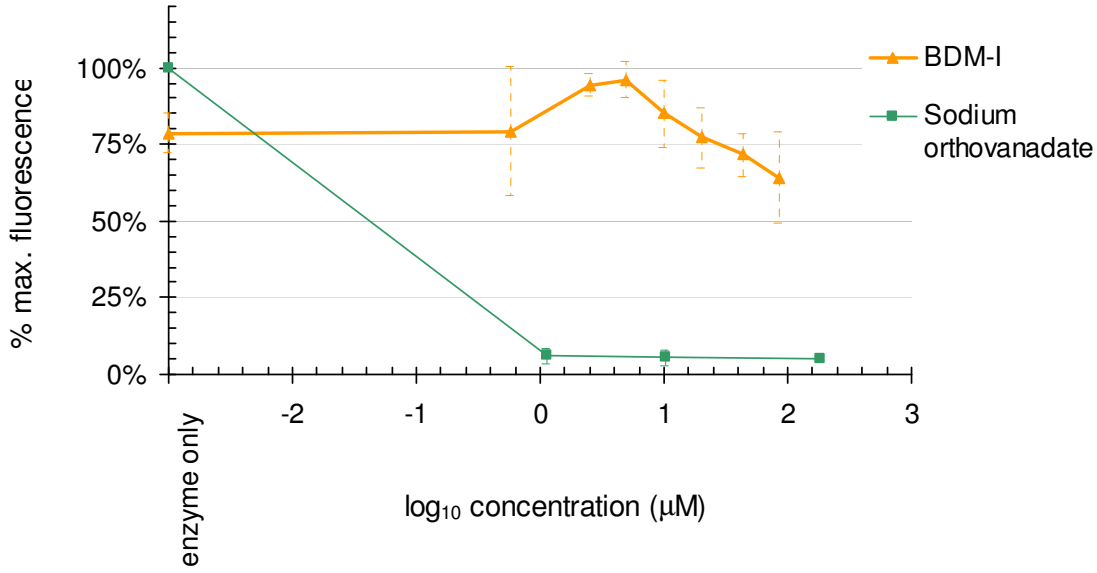


Figure 5-8. Effect of BDM-I and sodium orthovanadate on fluorescence of R110 substrate for PTP enzyme assay using CD45.

The amount of CD45 enzyme used was 5 U. Error bars represent standard deviation.

5.4.4 Discussion

Park & Pei (2004) have demonstrated that benzyl-β-nitroethene and some derivatives are competitive inhibitors of protein tyrosine phosphatases. In the assays reported here, BDM-I showed similar level of inhibition of PTP1B (IC_{50} 16 μM) to that reported by Park & Pei (Guo *et al.*, 2002) for a related benzyl nitropropene (Compound 6, 23 μg/mL). The inhibiting concentration against Yop was 25 μM and BDM-I was much less active against CD45. Inhibition of CD45 would have implications for host immunity. CD45 positively regulates the response of functional lymphocytes to stimulation through antigen receptors. The inhibitory effect of BDM-I would need to be investigated for a possible effect on T-cell function before therapeutic options could be discerned.

Characterisation of signalling networks in cells is a very active field in biomedical research. The catalytic site of PTP's is highly conserved and it has been difficult to develop selective PTP inhibitors (Huyer *et al.*, 1997). Thiol-oxidising agents, such as vanadate, are potent general PTP inhibitors. Vanadate, a phosphate analogue, is a reversible competitive inhibitor of PTP1B, oxidising the cysteine residue at the catalytic site (Combs *et al.*, 2005; Desmarais *et al.*, 1999; Taing *et al.*, 1999; Xian *et al.*, 2000; Zhang, 2002). Many PTP inhibitors are composed of a tyrosine mimetic that reacts with the PTP active site and a component peptide or peptidomimetic or small molecules that interacts with key residues near the active site (Lydon & Druker, 2004).

Protein tyrosine kinases have proven a successful target for anti-tumour agents such as imanitib (Zhang & Zhang, 2007). PTP inhibitors may also be useful therapeutic candidates for the treatment of cancer, diabetes, obesity and immune disorders. Several inhibitors of PTP1B are currently being investigated for therapeutic applications (Taing *et al.*, 1999; Zhang, 2001). BDM-I has potential as a lead

compound in the development of anti-infective agents based on PTP inhibition. Selectivity of action against bacteria is more likely given the differences between prokaryotic and eukaryotic PTP's in regions outside the active site (2004).

PTP inhibitors are useful compounds to investigate signal transduction pathways and the physiological functions of PTP's. There is a need for selective/specific, small molecule active site inhibitors that can enter cells. Potent inhibitors, such as the non-hydrolisable phosphotyrosine mimetics, tend to be highly charged with consequent low bioavailability. BDM-I is a neutral tyrosine mimetic and penetrates cell membranes. Peptide-containing analogues of BDM-I can be synthesised via a carboxyl substitution on the benzyl ring. Park & Pei (2004) showed that the addition of a tripeptide to benzyl nitroethene resulted in increased activity against PTP's.

More work is needed on the kinetics to see where slow-binding inhibition observed by Park & Pei (Brazas & Hancock, 2005; Freiberg *et al.*, 2004a) for related compounds against PTP's is also applicable to BDM-I.

CHAPTER 6 THE EFFECT OF BDM-I ON GENE EXPRESSION

6.1 INTRODUCTION

Genetic mapping of resistant mutants can help identify the MOA of a compound. Use of genetic analyses of resistant phenotypes to discover altered genes and proteins was not possible in this investigation since in repeated trials BDM-I failed to produce stable resistant phenotypes. A whole genome transcription expression analysis was therefore used to investigate the global changes in gene expression in *Bacillus subtilis* on exposure to BDM-I.

In living cells, environmental cues such as chemical stress, induce specific patterns of gene expression. Transcriptional profiling using DNA microarray analysis is a recognised tool for evaluating changes in gene expression due to inhibitory agents and characterising or validating a MOA (Brazas & Hancock, 2005). The bacterial gene expression profile responds very rapidly to environmental change with consequent changes in mRNA abundance (transcriptome). Genomic responses of organisms to an inhibitor are very complex. The gene expression profile will reflect changes that are a direct consequence of target inhibition, changes in genes that are indirectly affected by changes in the primary target and secondary changes resulting from the action of the compound. There may also be organism or strain-specific responses, such as those related to virulence mechanisms and changes in genes of unknown function (Freiberg *et al.*, 2004b; Hutter *et al.*, 2004a; Hutter *et al.*, 2004b; Ng *et al.*, 2003).

Transcriptional profiling of cells and conditional mutants treated with antibiotics has been used to identify characteristic profiles for well known classes of

antibacterial agents. Each class of inhibitor induces changes in distinct sets of genes (a gene signature) in particular species, which reflect the primary targets for that class of compound. Expression profiles induced by antibiotics with a known MOA in model organisms can be used to classify particular mechansims and to identify specific marker genes (2004b). These studies have shown that well known agents can have many targets and different mechanisms of action. Such reference compendia can be used to predict the MOA of novel compounds from comparison of their expression profiles with reference profiles. Hutter *et al.* (2004a) have compiled database of transcriptional profiles of *B. subtilis* for 37 well-characterised antibiotics as a prediction tool for the MOA of uncharacterised compounds. Hutter *et al.* (Freiberg *et al.*, 2004a) have also identified promotor regions which are markers for antibiotic targets and constructed reporter strains of *B. subtilis* as indicators of specific MOA.

B. subtilis is useful as a model organism for functional genomics-based drug research due to the abundance of physiological information available and its phylogenetic relationship to major pathogens such as staphylococci, enterococci and streptococci (Peterson *et al.*, 2001).

In this study Affymetrix Genechip® technology was used for a preliminary characterisation of the global expression responses of *Bacillus subtilis* treated with BDM-I focusing on concentration-dependent changes at one time point. The analysis sought to identify genes whose transcription levels were significantly changed and subject those to a functional evaluation using an annotated gene database for *B. subtilis* and, if possible, relate them to biological observations of the effects of BDM-I.

6.1.1 DNA Microarray analysis

Measurement of the trancriptome (mRNA levels) is based on the hybridisation of sample cDNA with DNA probes spotted on microarray chips. The

level of transcripts detected in a treated population is then compared to that of an untreated control. Computational algorithms and statistical analyses identify genes with significant differential expression and online annotational databases allow the categorisation of genes based on related functions.

Affymetrix GeneChip®

Affymetrix GeneChip® microarrays are created by using lithography to coat quartz slides with small fragments of chemically synthesised DNA. These fragments are termed probes and a probe set consists of one oligonucleotide with a perfect match and one with a single base mismatch. The location of each probe set is known as a feature, with each array containing millions of features. On antisense arrays the oligonucleotide sequence is the same as the coding sequence. Nucleic acids can then be extracted from samples and cDNA generated, labelled and hybridised to the probes on the array. Monitoring of the amount of label at each feature enables analyses of transcript levels indicative of expression and subsequent functional analyses.

The Affymetrix GeneChip® *B. subtilis* Genome Array consists of 5039 probe sets and enabling the relative monitoring of mRNA transcripts from 4,350 open reading frames and 600 intergenic regions from *B. subtilis*. The array contains 45 control probe sets and probes that are complementary to the labelled first strand cDNA that is hybridised to the array. Probe set data are available online through the NetAffx Feature on the Affymetrix website (www.affymetrix.com).

JCVI CMR Primary annotation database

Differentially regulated genes identified in treated samples through statistical analyses were categorised by functional roles based on the JCVI CMR online database (2004b). The J. Craig Venter Institute (formerly known as The Institute for Genomic Research, TIGR) Comprehensive Microbial Research (CMR) database (http://cmr.jcvi.org/tigr-scripts/CMR/GenomePage.cgi?org=ntbs01, June 2007) describes functional role categories for 2572 of the 4100 protein coding genes identified (Figure 6-1).

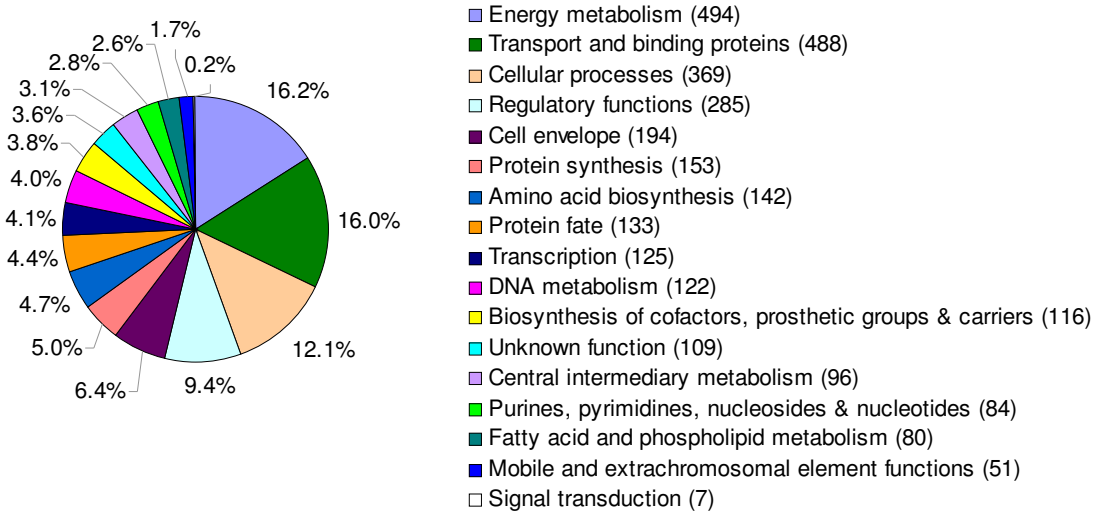


Figure 6-1. Main functional categories of *Bacillus subtilis* protein coding genes identified in the primary annotation JCVI CMR database.

Primary annotation of *B. subtilis* genome from the JCVI CMR database. Pie chart percentages indicate the percentage of the total 4100 protein coding genes categorised to the nominated function. Figures in parentheses indicate the total number of genes in functional categories.

6.2 MATERIALS AND METHODS

6.2.1 Selection of treatment parameters

There are as yet no strict guidelines for conducting and analysing microarray experiments. A number of people interested in the use of microarrays in antimicrobial MOA studies have suggested parameters for such investigations using *B. subtilis* including Hutter *et al.* (2005) and Freiberg *et al.* (Brazas & Hancock, 2005; Freiberg *et al.*, 2004a). These parameters, based on time kill and optical density data, were considered in choosing treatments for this study and were similar to those used in Hong *et al.* (2003). Treatment concentrations were determined as described below.

A broth dilution assay to determine the MIC of *B. subtilis* ATCC 6633 in AOAC broth were performed as described in Section 2.2.2 and gave an MIC of 16 µg/mL and an MMC of 32 µg/mL. A time-kill assay was performed as described in Section 2.6.2 in AOAC broth with $\log_2$ concentrations of BDM-I from 2 to 32 µg/mL to determine treatment concentrations. Cell densities, measured by viable counts using plate dilution and OD_{600nm}, were recorded at 0, 0.5, 1, 2 and 4 h. The time kill assay showed a similar rate of growth (70% of the control) for 2, 4, 8 and 16 µg/mL treatments to 30 min. Viable cell counts and optical density data for 2 and 4 µg/mL were similar.

The two treatment levels chosen for the microarray srudy were a subinhibitory concentration of 2 µg/mL and an inhibitory concentration of 16 µg/mL BDM-I to give a greater differential in treatment concentrations. The time point of 30 minutes was chosen as it is approximately half the generation time in AOAC broth. The sub-inhibitory dose and analysis of an early time point was used to minimise expression of secondary targets and downstream effects. Use of inhibitory concentrations allows detection of expression of genes outside the target pathway. (Affymetrix Inc., 2001).

Optical density data and viable counts were collected during treatment of samples to ensure close replication of sample treatments (Appendix B).

6.2.2 Total RNA isolation

B. subtilis was grown overnight in Synthetic broth AOAC (Difco™ Laboratories, USA) on a shaker (Ratek) at 160 rpm. This culture was diluted 1:100 in fresh medium and returned to the shaker. Bacterial growth was monitored by OD_{600nm} using a spectrophotomter (Cary 50 UV-Vis, Varian) and by dilution plates (Appendix B). At 3 h the culture was divided and BDM-I was added at 2 µg/mL or 16 µg/mL (1% DMSO). The control culture received the same volume of DMSO vehicle. Cell density was measured as above at 0 and 30 minutes. After 30 minutes, RNA was isolated using the Qiagen RNAprotect® bacteria kit, which includes a reagent that stabilises the RNA expression profile of the sample *in vivo* (Qiagen RNAprotect® bacteria reagent handbook, 2005). Protocol 5 was followed for enzymatic lysis, proteinase K digestion and mechanical disruption of the bacteria. Protocol 7 was followed for purification of total RNA from bacterial lysate using the RNeasy Mini Kit with on column RNase digestion. RNA was eluted in 2 × 30 µL of provided RNase free water. Samples were designated a number based on the replicate group (3, 6, 7) and a letter to indicate the treatment (a, untreated; b, 2 µg/mL BDM-I; c, 16 µg/mL).

Isolated total RNA was diluted in MilliQ water treated with 0.1% v/v diethylpyrocarbonate (DEPC) (Sigma-Aldrich) and autoclaved to remove RNase. Concentration was checked by spectrophotometry at 260/280 nm in quartz cuvettes with a path length of 1 cm (Cary 50 UV-Vis, Varian). A 260/280 ratio of 1.8-2 was considered optimal for labelling and hybridisation to the array. Concentrations were calculated and found to be 364-956 µg/mL with ratios 1.72-1.86 (Appendix B). The quality of RNA was checked by electrophoresing samples on a 1.2% formaldehyde

agarose gel at 80V as described in the RNeasy handbook (Appendix A). Ribosomal bands were visualised under UV light using a gel documentation system (Gel Doc, Bio-Rad). The total RNA was deemed acceptable for each of the 9 samples, as each produced clear bands and showed no degradation (Appendix B).

6.2.3 Labelling and hybridisation

Labelling of the 9 RNA samples from *B. subtilis* and hybridisation to GeneChips was carried out by the Australian Genome Research Facility (AGRF, Parkville, Vic, AUS) according to standard Affymetrix protocols. Briefly, the quality of *B. subtilis* total RNA was confirmed on the Agilent Bioanalyser 2100 using the NanoChip protocol (Supplemantary data CD). A total of 7 µg of each sample was used according to recommended GeneChip® *E. coli* Antisense Genome Array Expression Analysis Protocol (Affymetrix Inc., 2004).

GeneChip® Eukaryotic poly-A RNA spikes (Millenium Science, Vic, AUS) were added to the reaction to indicate the labelling efficiency. First strand cDNA was generated according to Affymetrix protocols and cleaned using the Affymetrix GeneChip® Sample Cleanup kit (Millenium Science). The cDNA was then fragmented using DNase I. A final quality check of the fragmented cDNA was performed on the Agilent Bioanalyser 2100 using the NanoChip protocol.

The fragmented cDNA was end-labelled using terminal deoxynucleotidyl transferase enzyme and the Affymetrix GeneChip® DNA biotinylated labelling reagent (Millenium Science). A total of 7 µg of labelled cDNA was then hybridised to the *B. subtilis* GeneChip® (Millenium Science) by preparing a probe cocktail (labelled cDNA, 0.05 µg/µL) that includes 1× Hybridisation Buffer (100 mM MES, 1 mM NaCl, 20 mM EDTA, 0.01 % Tween-20), 0.1 mg/mL Herring Sperm DNA, 0.5 mg/mL bovine serum albumin and 7 % DMSO.

A total hybridisation volume of 300 µL was prepared for each sample and 200 µL loaded onto each GeneChip®. The chip was hybridised at 45ºC for 16 h in an oven with a rotating wheel at 60 rpm. After hybridisation the chip was washed and stained with Streptavidin-Phycoerythrin (SAPE) using the appropriate fluidics script (ProkGE-WS2) in the automated Affymetrix Fluidics Station 450. Upon completion of washing, the chips were scanned at 570 nm using the Affymetrix GeneChip® Scanner 3000. The scanner operating software, GCOS, converted signals on the chips into image files (*.DAT). Image files were received from AGRF for analysis using GCOS software (Supplementary data).

6.2.4 Analysis of data

Image analysis (GCOS)

The global method was used to generate a grid, anchored at each corner of the array and based on the feature size of 18 µm to determine the pixel coordinates for each feature. Each raw image file (*.DAT) was inspected carefully for successful hybridisation of the B2 oligo border, correct alignment and for image artefacts. All arrays were satisfactorily aligned as indicated by the alternating pattern. Outlier cells (fluorescent intensity) detected by GCOS and cells affected by system artefacts detected visually were ‘masked’ and eliminated before cell intensity (*.CEL) files were generated. Artefacts were only observed on one array out of nine (6a, untreated). System artefacts may be due to array manufacture itself, application of hybridisation cocktail or washing and staining procedures.

Cell intensity image files were used to generate probe set data (*.CHP) and report files for each array to provide data for absolute analysis.

Absolute analysis (GCOS): Evaluation of data quality

Scatter plots of signal values for treatment versus control data were generated from probe set data (*.CHP files) and examined for skewness and variability (Figure 6-2). This single array analysis included examination of a number of factors across replicates including scale factor, background, noise and percentage of probe sets called 'present' (Table 6-3). Values for control probe sets (spike controls, polyA tails, internal housekeeping genes) were also examined for each array.

Comparison analysis (GCOS): Experiment versus baseline arrays for each replicate

The data was filtered using criteria based on detection calls, change calls and signal log ratios generated using the GCOS program to provide an overview of changes between treated and untreated cells.

Comparison analyses between control and treated samples within each replicate (*.CEL files), were performed in GCOS for first order analyses of expression differences. For each biological replicate the untreated control was set as the baseline file for comparison to each BDM-I treatment in that replicate and .CHP file data generated including signal log ratios, change calls and change *p*-values.

The detection algorithm in GCOS calculates a discrimination score (R) from probe pair intensities according to the formula:

$$R = (PM-MM)/(PM-MM)$$

where PM is the incidence of perfect match probe pairs and MM is the incidence of Mismatch probe pairs. The discrimination score is compared to a user defined value, Tau (0.015) and the probe set assigned a detection call of "Present" if R>Tau or "Absent" if R<Tau. The confidence of this calculation is reflected by a *p*-value which is generated by a One-sided Wilcoxon's Signed Rank test and refines the detection call

based on the α1 (0.04) and α2 (0.06) parameters (Appendix C). Probe sets are assigned a final detection call of 'Present' if p= <α1, marginal if p=α1>α2 and absent if p=>α2. Signal is then calculated by a One-step Tukey's Biwight Estimate and represents relative expression levels (1995). The program compares PM-MM intensities and assigns a change call as I (increase), D (decrease) or NC (no change) with an associated p-value. The signal represents relative abundance of the transcript and the signal log ratio indicates the direction of the change call and its magnitude. Significantly regulated genes identified from comparison analyses for each BDM-I treatment concentration were selected in GCOS by the criteria listed in Table 6-1.

Table 6-1. Criteria for selection of significantly regulated genes in GCOS in comparison analysis.

Up-regulated genes	Down-regulated genes
Detection call of "Present" in treatment	Detection call of "Present" in untreated (baseline)
Change call of "Increase"	Change call of "Decrease"
Signal log ratio ≥1 (i.e. ≥2-fold change)	Signal log ratio <1 (i.e. <2-fold change)
Signal Units >100	Signal Units >100

Present calls are based the discrimination score generated from probe match intensities and compared to user definable parameters, Tau (0.015), α1 (0.04) and α2 (0.06).
Change calls are based on probe match intensities and reflected by the signal log ratio. Signal units reflect the relative abundance of the transcript.

Statistical analysis of microarray data

Analysis of microarray data was performed on data exported into an Excel spreadsheet (Microsoft®, WA, USA, 2003). Imported text files of probe set data were added as two datasets each consisting of three replicates of controls and either 2 µg/mL or 16 µg/mL treatments. Altered transcript levels for probe sets for treatment samples compared to the untreated control in each replicate were identified by performing a

paired t-test at a confidence level of 95%. Only those probe sets which were differentially regulated (P<0.05) in 2 of 3 biological replicates of treatments were selected for further analysis. Probe sets with a mean signal of <100 in both untreated and treated samples were discarded. Probe sets with a mean signal of <100 in the untreated samples were discarded. Significantly changed probe sets were considered to have a fold change of ≥2. Significantly changed probe sets were ranked according to ascending *p*-values and a Benjamini and Hochberg (Ko *et al.*, 2004) false discovery rate (FDR) was applied to isolate the final list of probe sets of interest. The application of this FDR is one of the least stringent statistical methods for evaluating false positives and negatives and tolerates more false positives with the advantage that it identifies less false negatives. The method involved ranking *p* values in ascending order and the FDR was calculated according to the equation: *p* value × total number in list/rank with a cut-off of 0.05.

Functional analysis of differentially regulated genes

The genes represented by these final probe set lists were then placed into functional categories based on data current in the online JCVI CMR database. Primary Annotations were used to identify functional categories for *B. subtilis* genes and to describe differentially regulated genes in response to BDM-I exposure demonstrated by changes in trancripts levels.

6.2.5 Validation of microarray results by quantitative PCR

Validation of microarray data performed by qPCR on original samples provided experimental verification of gene-expression levels.

RNA samples used for the microarray were used to generate cDNA using Superscript™ II reverse transcriptase (Invitrogen Australia, Vic, AUS). A total of 5 ng of total RNA was combined with 225 ng random primers (Invitrogen Aust.), 1 µL dNTP mix (10 mM each, Invitrogen Aust.) and nuclease free water (Promega) to 12 µL. The mixture was heated to 65°C for 5 min using a thermocycler (PCR Express, Hybaid, MA, USA) and quickly chilled on ice. The contents of each tube was collected by brief centrifugation and 5× first strand buffer (4 µL), 0.1 M DTT (2 µL) and 40 U/µL RNaseOUT (Invitrogen Aust.) (1 µL) added. Tube contents were mixed briefly and incubated for 25°C for 2 min. Superscript II RT (200 U) was added and mixed by pipetting and the tubes incubated at 25°C for 10 min and at 42°C for 60 min. The reaction was inactivated by heating at 70°C for 15 min. First strand cDNA was purified using Qiaquick spin columns (Qiagen, Vic, AUS) according to manufacturer's instructions. Final elution was in two volumes of 50 µL of buffer EB for a final concentration of 50 ng/µL.

Changed probe sets common to both treatment levels were identified and are shown in Figure 6-5. Three were selected for validation of microarray results using qPCR with sequence specific primers. Target genes were *clpC* (a class III stress response-related ATPase), *spoVG* (required for stage V sporulation) and *dnaE* (DNA polymerase III subunit alpha). One unchanged gene, *mbl*, was included as an internal housekeeping control. It encodes an actin-like protein and has been used in other studies as a housekeeping control in *B. cereus* (Altschul *et al.*, 1990).

Sequence specific primers were designed based on probe set sequences from Affymetrix. Primer design was carried out online using PrimerQuest from Integrated DNA technologies (http://www.idtdna.com/Scitools/Applications/Primerquest/). Primer sequence specificity was checked using the basic local alignment search tool (blastn,

NCBI, (Pfaffl, 2001). Primers were synthesised by Geneworks (SA, AUS). Stocks were prepared at 10 µM from the lyophilised sequences in Nuclease free water (Promega) and stored in aliquots at -20°C. A summary of the primers used is shown in Table 6-2.

Quantitative PCR reactions were carried out using SYBR green supermix (Bio-Rad Laboratories) and the Miniopticon™ real-time PCR detection system in a thermal cycler (MJ Mini™, Bio-Rad). Validation of primer sets was performed using 2-fold amounts of template equivalent to 12.5 ng up to 400 ng starting material for cDNA first-strand synthesis. Melt-curve analysis and agarose gel electrophoresis was used to confirm generation of distinct PCR products (data not shown).

The following cycling protocol was used: denaturation (95°C, 10 min) amplification and quantification repeated 35 cycles (95°C, 15 s; 55°C, 20 s; 72°C, 20 s, single fluorescence measurement), 95°C, 1 min, cooling at 50°C, 1 min, melting curve program (50-99°C with heating rate of 1°C per 5 s and a continuous fluorescence measurement, cooling step to 4°C.

Standard curves consisting of two-fold sample concentrations in duplicate were used to determine primer pair efficiencies for each gene. Efficiencies were calculated from the C_t using the pearson correlation coefficient according to the equation $E = 10^{(-1/slope)}$ and are presented as percentages in Table 6-2.

Table 6-2. Summary of primers used for validation of microarray results by qPCR.

Probe Set ID	Primer Name	Sequence 5' → 3'	Target length	Start	T_m (ºC)	E (%)
BG10916/mbl_at	mbl F	CAAACGCGAGTACAAGCTGCTGAT	631	300	59.6	76
	mbl R	AAATTTCCTCGTGACGTGCGTCTG	631	397	59.5	
BG12583/dnaE_2_at	dnaE_2 F	TTATGTGCGGATTGCTGACAAGCG	1261	633	59.9	76
	dnaE_2 R	TGACTGCTGAGACGCCTACACTTT	1261	824	59.9	
BG10112/spoVG_at	spoVG F	GCATGAGAGCGATTGCATCCATCA	271	29	59.5	93
	spoVG R	CGTTTACTCGGCATCGCAACGAAA	271	134	59.9	
BG10148/clpC_at	clpC F	GAACTTGCACGAGCACTTGCTGAA	2173	1582	60.0	90
	clpC R	ACATAACCCGGAGGTGAACCAACA	2173	1706	60.1	

F = forward primers (same sequence as probe); R = Reverse primers (reverse complement of the probe); E = efficiency of primer pair according to Pearson's correlation coefficient.

The cycle threshold (C_t) is defined as the point at which the fluorescence rises appreciably above background fluorescence. The cut-off threshold value for calculation of the C_t in experimental reactions was set manually at the same level at which maximum efficiency was achieved in the standard curve for each set of primers.

Quantitative PCR was performed on three seaparate occasions. Average relative fold changes for each treatment were calculated using the Pfaffl method with reference to the housekeeping gene (*mbl*) and the untreated control (Hong *et al.*, 2003). The equation for calculating the Relative Expression Ratio is shown below:

$$\frac{(E_{target})^{\Delta Ct_{target}\,(Ct_{untreated} - Ct_{treated})}}{(E_{housekeeping})^{\Delta Ct_{housekeeping}\,(Ct_{untreated} - Ct_{treated})}}$$

where E is the expression of a gene calculated from Ct (cycle threshold) values of genes across samples.

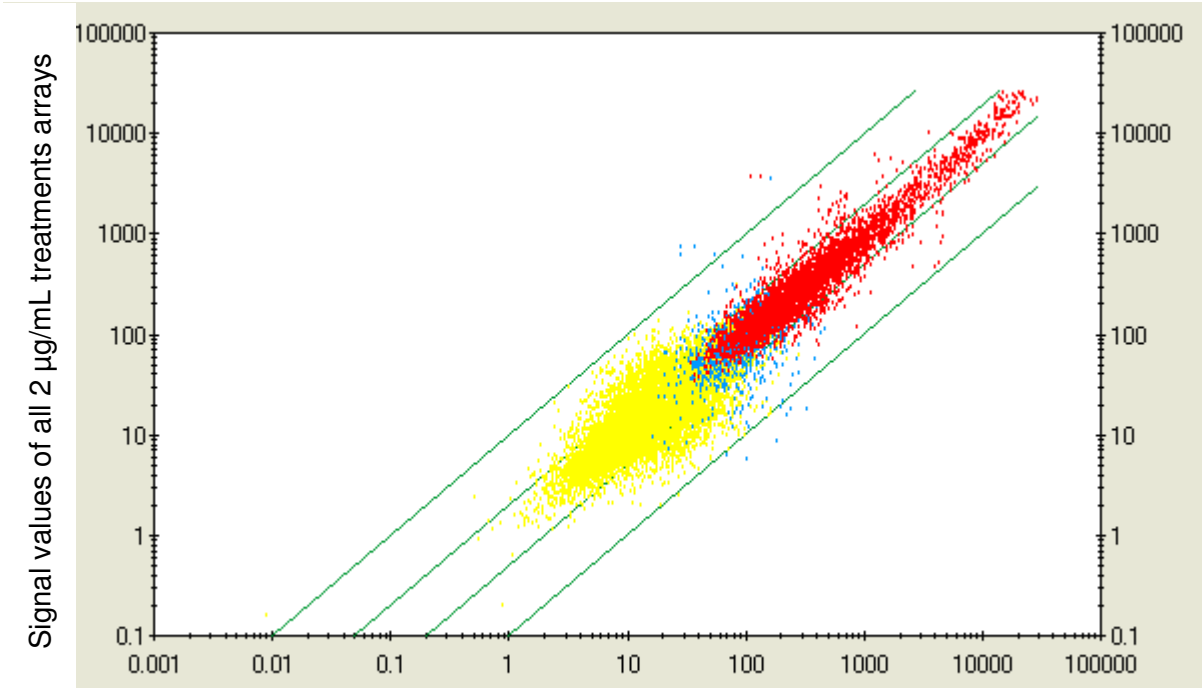
6.3 RESULTS

6.3.1 Absolute analysis: Evaluation of data quality

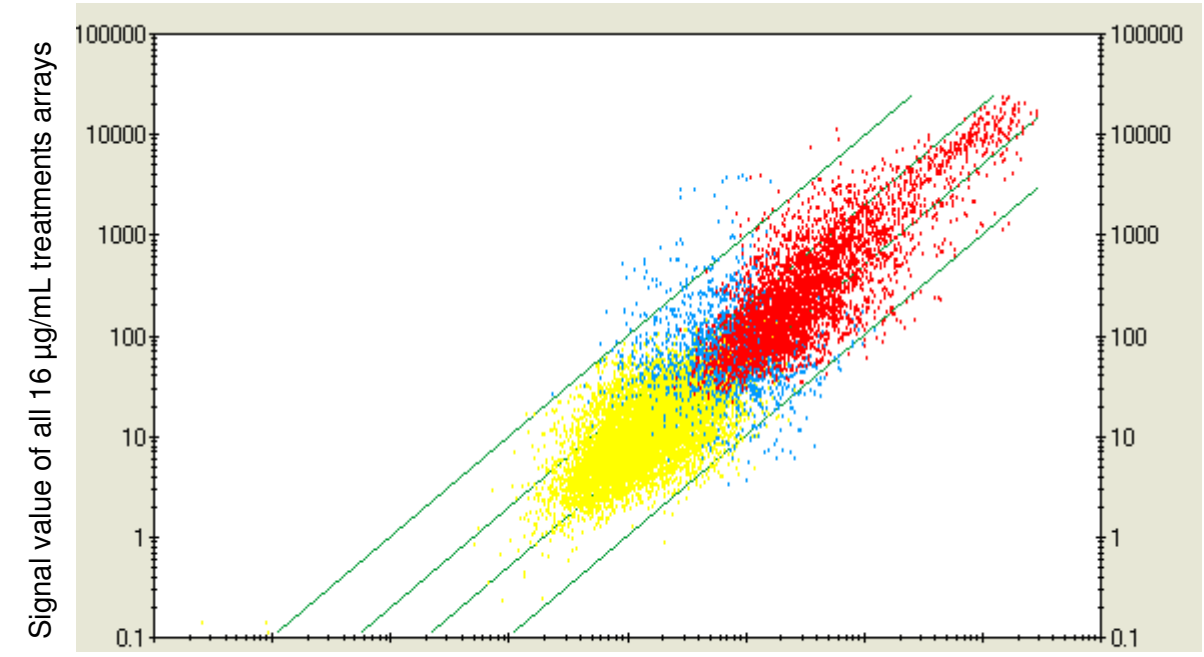
The scatter plots enable visualisation of comparisons between all biological replicates of arrays within treatments (Figure 6-2). These graphs show that the data points are not skewed for either treatment level indicating that the data represents reliable biological replicates.

The parameters used to assess the quality of the microarray data presented in Table 6-3 indicate acceptable replicate data was obtained from the samples on separate gene chips. The scale factor for all arrays was well within the 3-fold acceptable range and ruled out the need to normalise signal intensities to the baseline before comparison analyses. The mean average background signal range of 96.0 to 170.8 was outside the normal range of 80-130 which applies to the new scanning technology at the AGRF facility. The discrepency was deemed to be caused by the mismatch of technology as the *B. subtilis* chip, is available only in an older platform version. The background range obtained was deemed acceptable by AGRF. Both the background and the RawQ values were acceptable, indicating that the image quality was satisfactory.

The proportion of the probe sets with a detectioin call of 'present' in this analysis varied from 27% to 39% with a mean of 32.5%. This is comparable to similar gene chip studies on *E. coli* (Kobayashi *et al.*, 2003).



a)



b)

Figure 6-2. Scatter plots for *B. subtilis* treatment signals against control group signals.

B. subtilis was treated with 2 µg/mL BDM-I (a) or 16 µg/mL BDM-I (b). Lines represent 2-fold and 10-fold differences respectively. Each dot represents a probe set. Colours represent detection calls between probe pairs. Key: Red, both Present (PP); Blue, one Present (MP, PM, AP, PA); Yellow = None Present (MM, AM, MA, AA).

The hybridisation efficiency controls, biotin labelled spike controls (BioB and Cre) increased in the expected order indicating that hybridisation efficiency was satisfactory. However, the lysine spike control did not give expected signals on any of the arrays and only one Poly A tail labelling control was detected as present for all arrays. This is due to the prokaryotic sample not possessing polyadenylated tails and is required predominantly on eukaryotic arrays as a hybridisation control. The 3'/5' ratio for housekeeping genes was <3 in all instances indicating good RNA integrity, efficient first strand cDNA synthesis and transcription of cRNA.

Table 6-3. Results of absolute analysis of data quality parameters for *B. subtilis* GeneChip® experiments.

Attribute	Treatment: Untreated cells			Low concentration (2 µg/mL)			High concentration (16 µg/mL)				
Replicate i.d.	*3a*	*6a*	*7a*	*3b*	*6b*	*7b*	*3c*	*6c*	*7c*	**Mean**	***s.d.***
Raw Q noise[1]	3.3	2.5	2.5	3.2	3.0	2.9	3.1	3.0	2.3	**2.9**	***±0.4***
Average Noise	17.2	9.9	10.5	15.8	12.7	13.7	14.3	13.4	8.3	**12.9**	***±2.9***
Average Background[2]	170.7	108.6	113.5	163.3	139.7	138.7	156.6	147.7	96.0	**137.2**	***±25.9***
Scale factor[3] at TGT 150[4]	1.4	2.1	1.4	1.9	1.6	1.6	0.8	1.1	1.8	**1.5**	***±0.4***
% genes present[5]	28.8	30.7	35.2	26.9	31.4	30.9	38.5	35.7	34.2	**32.5**	***±3.7***
Average % present		31.6			29.7			36.1			

[1]Raw Q noise and Average noise values measures the degree of pixel-to-pixel variation. Replicates should be within 5 RawQ noise units.

[2]Mean average background expected range is 80-130 but may vary with Genechip® platform and scanner technology employed.

[3]Scale factor indicates closeness of average array intensity to the target intensity, 1.0 indicating equal intensity and a 3-fold range being acceptable.

[4]Target signal value = 150 is Affymetrix recommendation for the AGRF protocols and equipment.

Percent genes called 'present' from 5039 probe sets.

Appendix D

Variation coefficients for probe set signals for all biological replicates were calculated by dividing the standard deviation by the mean signal and presented as a percentage to demonstrate reproducibility across biological replicates (Figure 6-3). In total, over 98% of genes called present for at least 2 of 3 treatment replicates had variation coefficients (VC) less than 50%.

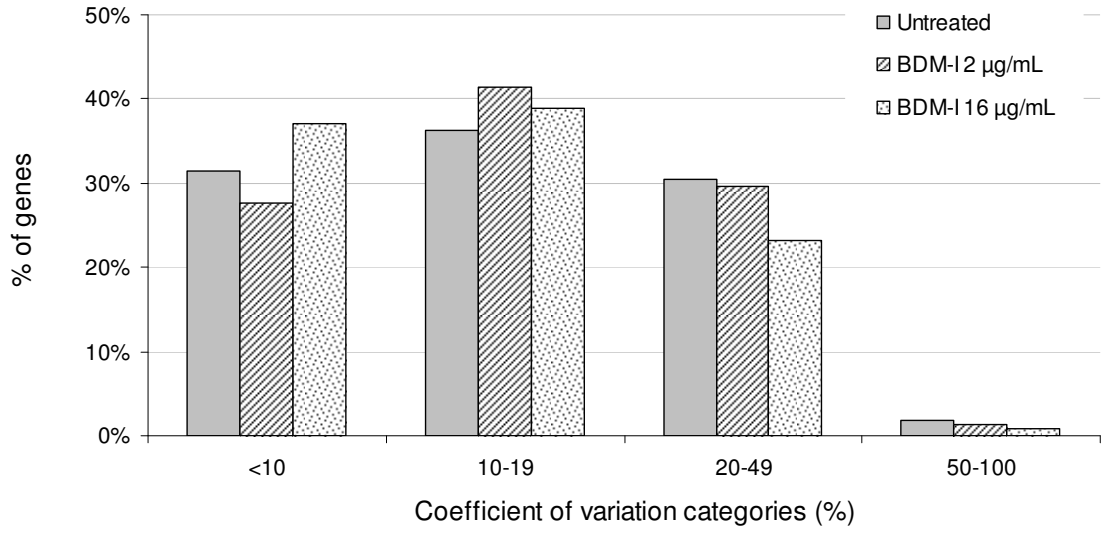


Figure 6-3. Reproducibility of Genechip® signal data across treatment groups.

VC % of genes called present for at least 2 of 3 biological replicates for each treatment. VC % = stdev/mean *100.

6.3.2 Comparison analysis: Experiment versus baseline arrays for each replicate.

Comparison analyses, using GCOS software (ver 1.0, Affymetrix Inc.) and applying the selection criteria in Table 6-1, between each treatment and the control (pairs) for each biological replicate identified probes sets showing increased or decreased expression (Table 6-4). The comparison analysis showed consistency of data across biological replicates.

Table 6-4. Percentage of probe sets changed in *B. subtilis* treated with BDM-I compared to controls for each biological replicate performed in GCOS.

	Low concentration (2 µg/mL)				High concentration (16 µg/mL)			
Replicate i.d. (baseline & treatment)	*3a&3b*	*3a&3b*	*6a&6b*	**mean**	*7a&7c*	*6a&6c*	*7a&7c*	**mean**
Increases								
Number of probe sets 'P'	1351	1579	1554	**1495**	1936	1797	1721	**1818**
Also with a Change call of 'I'	233	266	176	**225**	547	557	509	**538**
Also with a Signal Log Ratio of ≥1	71	45	65	**60**	281	290	285	**285**
Also with signal >100	69	44	64	**59**	266	276	263	**268**
% Probe sets increased	5%	3%	4%	**4%**	14%	15%	15%	**15%**
Decreases								
Number of probe sets 'P' in Baseline	1450	1542	1773	**1588**	1450	1542	1773	**1588**
Also with a Change call of 'D'	290	252	337	**293**	575	475	712	**587**
Also with a Signal Log Ratio of ≤-1	79	63	45	**62**	507	222	449	**393**
Also with signal >100	58	36	19	**38**	233	36	184	**151**
% Probe sets decreased	4%	2%	1%	**2%**	16%	2%	10%	**10%**

Present calls are based the discrimination score generated from probe match intensities and compared to user definable parameters, Tau (0.015), $\alpha 1$ (0.04) and $\alpha 2$ (0.06).
Change calls are based on probe match intensities and reflected by the signal log ratio. Signal units reflect the relative abundance of the transcript.
Appendix D

6.3.3 Statistical analysis of microarray data

Data was exported from GCOS as text files and opened with Excel (Microsoft®, 2003) in order to perform statistical analysis (Supplementary data). A summary of the number of probe sets found changed by statistical analysis included application of a t-test and FDR and identified a number of probe sets significantly changed in response to BDM-I at 2 µg/mL and 16 µg/mL compared to the control (Table 6-5). Approximately 1% and 8% of the 5039 probe sets were significantly changed (up or down-regulated) in response to BDM-I at 2 µg/mL and 16 µg/mL respectively.

Forty six genes were changed at the sub-inhibitory concentration (2 µg/mL), 63% of these being up-regulated. Eight-fold more genes (384) were changed at the inhibitory concentration of 16 µg/mL with 66% of these being down- regulated. This suggests a strong adaptive stress response to BDM-I exposure.

Table 6-5. Probe sets identified as significantly changed in *B. subtilis* treated with BDMI at 2 or 16 µg/mL by sequential application of selection criteria based on statistical analysis of Genechip® data.

Statistical criteria	2 µg/mL treatment	16 µg/mL treatment
Total probe sets	5039	5039
Total probe sets differentially regulated by T-test ($p<0.05$)	582	1323
Total probe sets differentially regulated in at least 2 of 3 biological replicates	296	790
Total probe sets differentially regulated at least 2 of 3 biological replicates and with a mean signal >100 for both treatment and control	283	760
Total probe sets differentially regulated at least 2 of 3 biological replicates and with a mean signal >100 for control	273	743
Total probe sets significantly differentially regulated (fold change >2)	54	476
Total probe sets significantly differentially regulated after FDR applied	53	476
Total probe sets significantly differentially regulated after FDR applied and with "Present" calls for treatment in at least 2 of 3 biological replicates	46	384
Significantly up-regulated probe sets	**29**	**129**
Significantly down-regulated probe sets	**17**	**255**

6.3.4 Functional analysis of differentially regulated genes

The genes represented by the probe sets determined to be significantly up or down-regulated were identified in the JCVI CMR Primary annotation database (JCVI). Identified genes were grouped according to their functional categories for each treatment to present a holistic picture of transcriptional changes in response to exposure to BDM-I. The relevant probe lists are annotated to molecular and biological processes. Pie charts are used to represent the frequency of the identified functional categories.

Significantly changed genes associated with good microbial targets were the focus of this analysis.

Comparison of functional categories of genes

A breakdown of all primary annotated genes in the *B. subtilis* genome is shown in Figure 6-1. Functional categories are assigned to 2572 of the 4100 (63%) protein coding genes in the Primary annotation database.

Genes identified in this study that were not assigned to any functional categories were excluded from the pie charts while some genes have been assigned more than one main functional role. The percentages of differentially regulated genes identified as differentially regulated but not assigned functions (not found) in the JCVI CMR database were 16% (8 genes) and 27% (106 genes) for 2 μg/mL and 16 μg/mL BDM-I treatments respectively.

There are notable changes in the expression of functional gene categories between treated samples compared to those in the annotated JCVI database (Figure 6-4). This suggests the changes are a response to treatment with BDM-I and also indicates the rapidity of the adaptive response at the genome level to chemical stress. Compared to the annotated genome, for the sub-inhibitory (2 μg/mL) concentration, some categories are from 50% to ≥100% over-represented as a percentage of categorised genes. 'Energy metabolism', 'amino acid biosynthesis' and 'intermediary metabolism' are ~50% over-represented and 'biosynthesis of cofactors, prosthetic groups and carriers', 'purines, pyrimidines, nucleosides and nucleotides' and 'protein fate', more than 100 % over-represented. Two category functions are not represented at all in the differetially regulated subset: 'protein synthesis' and 'mobile and extrachromosomal element functions'.

For the higher concentration (16 μg/mL) more gene categories were identified and the differences in percentage representation compared to the annotated database were not as great. 'Purines, pyrimidines, nucleosides and nucleotides' and 'protein synthesis' are 100% over-represented and 'signal transductioon' ~50% over-represented.

The changes in functional category representation observed between the sub-inhibitory and the inhibitory concentration of BDM-I (8-fold concentration range) were variable. Most categories were under-represented at 16 μg/mL, but differential changes in 'protein synthesis', 'cell envelope' and transcription' were at least 7-fold higher at 16 μg/mL.

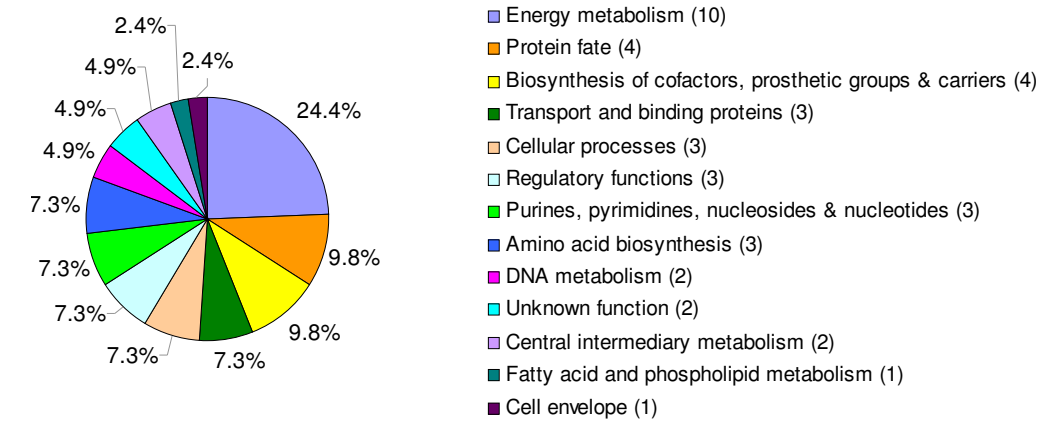
Comparison of differentially expressed genes

Functional annotations of significantly changed genes (up-regulated and down-regulated combined) is shown in Figure 6-4a & b.

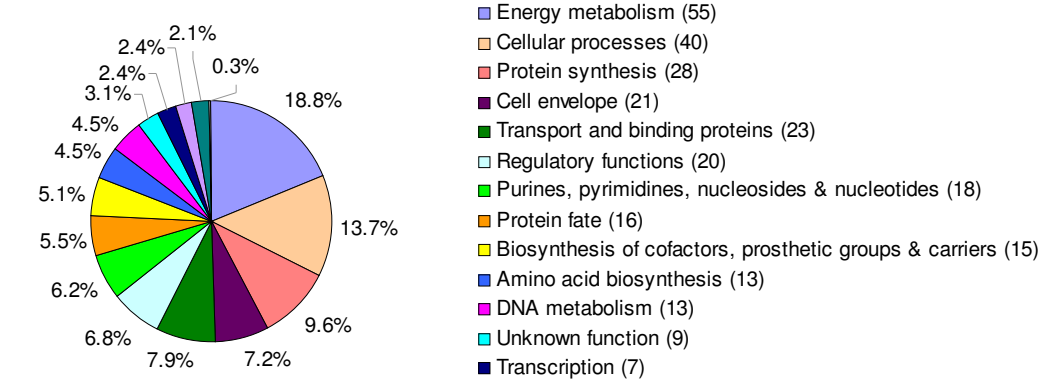
Of the 46 genes changed at 2 μg/mL, 10 related to energy pathways and 5 have unknown functions (2 being genes described as such in the JCVI database). The remaining genes were distributed fairly evenly over 11 functional areas.

Of the 384 genes changed at 16 μg/mL, 292 were spread among 15 of the 16 functional categories with 52.7% of these in the five largest functional groups (Figure 6-1). 'Energy metabolism' and 'Cellular processes' accounted for 55 and 40 changed genes respectively. Nine of the 292 genes are classified as 'unknown' function in JCVI.

Thirty-four differentially regulated genes have been identified as being essential to the survival of *B. subtilis* (Kobayashi *et al.*, 2003). Thirty-two of these were



a)



b)

Figure 6-4. Functional categories of differentially expressed genes in *B. subtilis* exposed to BDM-I at 2 µg/mL (a) and 16 µg/mL (b).

Primary annotation of *B. subtilis* genome from the JCVI CMR database. Data collected from a Genechip® microarray study described in Section 6.2. Figures in parentheses indicate number of changed genes (up-regulated and down-regulated) for treated samples in main functional categories.

changed in response to 16 µg/mL BDM-I. Of these, 9 are involved in protein synthesis (ribosomal proteins and tRNA synthases) and 5 are involved in energy metabolism. Twenty-three were down-regulated and 8 were up-regulated exclusively in response to 16 µg/mL BDM-I.

Genes differentially regulated at both treatment concentrations

To identify possible genes involved in the MOA of BDM-I, significantly changed genes that were common to both 2 and 16 µg/mL treatments were selected for functional analysis. BDM-I treatment at 2 µg/mL for 30 min reduced the cell numbers of *B. subtilis* by about 10%. The expression profile 2 µg/mL, therefore, better reflects the early changes due to the effect of BDM-I rather than follow-on effects or secondary effects due to greater growth arrest at 16 µg/mL.

Forty-six genes were differentially regulated at 2 µg/mL and 31 of these (67%) were also changed at 16 µg/mL. The commonly regulated genes constitute 8% of those changed at 16 µg/mL. Twenty up-regulated genes (≥2-fold change) common to both concentrations are shown in Table 6-6 and Figure 6-5a. The function of the most up-regulated gene (28-fold), *ycnE*, unfortunately has not yet been identified. Nine of these genes were in the top four represented categories in the normal *B. subtilis* genome and are involved in major cell activities (Figure 6-1). Of the genes changed in both treatments, only two, *clpP* (protein degradation) and *yugJ* (energy & intermediary metabolism), failed to show a larger change at the higher concentration. Three genes with lower representation in the reference genome, yraA (purines, pyrimidines, nucleosides & nucleotides), *yvrD* (fatty acid & phospholipid metabolism) and *yqfY* (biosynthesis of cofactors, prosthetic groups and carriers) have not been fully characterised and are listed as genes 'similar to known or hypothetical proteins'. One

gene, *dnaE*, which is involved in DNA metabolism and essential to B. subtilis survival was commonly up-regulated both both 2 and 16 µg/mL. Two genes, *trxA* and *trxB,* were differentially regulated in response to 2 µg/mL and 16 µg/mL BDM-I. Both are involved in energy metabolism and have been found to be essential *to B. subtilis* survival (Hutter *et al.*, 2004a).

Eleven genes are down-regulated at both concentrations (Table 6-7 and Figure 6-5). The most down-regulated gene, *spoVG*, was decreased 7-fold and 17-fold in 2 and 16 µg/mL treatments respectively. This gene is required for synthesis of the spore cortex. Two additional genes involved in spore formation were differentially expressed, *soj* (transcription regulator of spore proteins) which was down-regulated 2-fold at 16 µg/mL only and *yrbC* (spore coat protein) which was up-regulated 3-fold at 16 µg/mL only. Five down-regulated genes are involved in energy metabolism, one in regulatory functions, one in amino acid biosynthesis and three with 6-fold to 4-fold decreases, have no known function.

No commonly regulated genes were found that were up-regulated in one concentration and down-regulated in another. No commonly deregulated genes were involved in protein synthesis, cell envelope function and RNA transcription, all major targets of antibiotics. Promotor regions have been identified from transcriptional data in *B. subtilis* treated with a range of antibiotics and incorporated in reported strains which can be used as indicators of antimicrobial MOA (2001). None of these genes (*fabHB, glpD, yrzI, expZ, ypbG, ytrA, ywoB, dinB, yneA, yorB, ydeK, yvgS*) were found to be significantly changed by treatment of *B. subtilis* with BDM-I.

Table 6-6. Genes commonly up-regulated at both 2 and 16 µg/mL treatment concentrations of BDM-I.

Probe set ID	Gene Symbol	Fold change 2 µg/mL	Fold change 16 µg/mL	Main role	Sub role	Common Name
BG12041/ycnE_at	ycnE	28.6	28.2	not found	not found	not found
BG10146/yacH_at	yacH	5.4	10.1	Unknown function	General	similar to hypothetical proteins
BG10148/clpC_at	clpC	4.1	6.9	Protein fate	Degradation of proteins, peptides & glycopeptides	class III stress response-related ATPase
BG10145/ctsR_at	ctsR	4.5	6.2	Regulatory functions	DNA interactions	transcriptional regulator
BG11187/yckK_at	yckK	3.3	6.6	Transport and binding proteins	Amino acids, peptides and amines	similar to glutamine ABC transporter (glutamine-binding protein)
BG10147/yacI_at	yacI	3.6	5.7	Transport and binding proteins; Cellular processes	Carbohydrates, organic alcohols and acids; Toxin production and resistance	similar to creatine kinase
BG11186/yckJ_at	yckJ	2.1	6.7	Transport and binding proteins	Amino acids, peptides and amines	similar to glutamine ABC transporter (permease)
BG12583/dnaE_3_at	dnaE	2.7	5.5	DNA metabolism	DNA replication, recombination & repair	dna polymerase iii alpha subunit
BG12583/dnaE_2_at	dnaE	2.2	5.0	DNA metabolism	DNA replication, recombination & repair	dna polymerase iii alpha subunit
BG19016/clpP_at	clpP	4.3	3.3	Protein fate	Degradation of proteins, peptides & glycopeptides	ATP-dependent Clp protease, proteolytic subunit
BG12398/trxB_at	trxB	2.8	4.3	Energy metabolism	Electron transport	thioredoxin reductase
BG13776/yraA_at	yraA	2.7	3.8	Purines, pyrimidines, nucleosides & nucleotides	Pyrimidine ribonucleotide biosynthesis	similar to hypothetical proteins
BG14144/yvrD_at	yvrD	2.8	3.4	Fatty acid & phospholipid metabolism	Biosynthesis	similar to ketoacyl-carrier protein reductase
BG13023/yhdQ_at	yhdQ	2.5	3.7	Regulatory functions	DNA interactions	similar to transcriptional regulator (MerR family)
BG13559/yojG_at	yojG	2.7	3.0	not found	not found	not found
BG12364/yugJ_at	yugJ	3.2	2.4	Energy metabolism; Central intermediary metabolism	Electron transport; Fermentation; Glycolysis/gluconeogenesis; TCA cycle; Other	similar to NADH-dependent butanol dehydrogenase
BG13434/ymzA_at	ymzA	2.1	3.2	not found	not found	not found
BG13022/yhdP_at	yhdP	2.0	2.9	Cellular processes	Other	similar to hemolysin
BG10348/trxA_at	trxA	2.1	2.5	Energy metabolism	Electron transport	thioredoxin
BG11671/yqfY_at	yqfY	2.0	2.0	Biosynthesis of cofactors, prosthetic groups and carriers	Other	similar to peptidoglycan acetylation

Table 6-7. Genes commonly down-regulated at both 2 and 16 µg/mL treatment concentrations of BDM-I.

Probe set ID	Gene Symbol	Fold change 2 µg/mL	Fold change 16 µg/mL	Main role	Sub role	Common Name
BG10112/spoVG_at	spoVG	6.9	17.4	Cellular processes	Other	required for spore cortex synthesis (stage V)
BG13246/yknZ_at	yknZ	2.2	6.3	not found	not found	not found
BG10272/odhA_1_at	odhA	2.1	6.2	Energy metabolism	TCA cycle	2-oxoglutarate dehydrogenase (E1 subunit)
BG11156/yxjG_at	yxjG	2.2	5.8	not found	not found	not found
BG12681/sucD_at	sucD	3.2	3.7	Energy metabolism	TCA cycle	succinyl-CoA synthetase (alpha subunit)
BG13416/ymaH_at	ymaH	2.5	4.7	Regulatory functions	Other	similar to host factor-1 protein
BG11570/ybbI_at	ybbI	2.1	3.8	Unknown function	General	similar to hypothetical proteins
BG10673/ilvA_at	ilvA	2.2	3.5	Amino acid biosynthesis	Other	threonine dehydratase
BG10722/rocD_at	rocD	2.1	2.9	Energy metabolism	Amino acids & amines	acetylornithine aminotransferase
BG10353/sdhB_at	sdhB	2.3	2.7	Energy metabolism	TCA cycle	succinate dehydrogenase (iron-sulfur protein)
BG10478/citB_1_at	citB	2.2	2.5	Energy metabolism	TCA cycle	aconitate hydratase

not found – not listed in the JCVI database

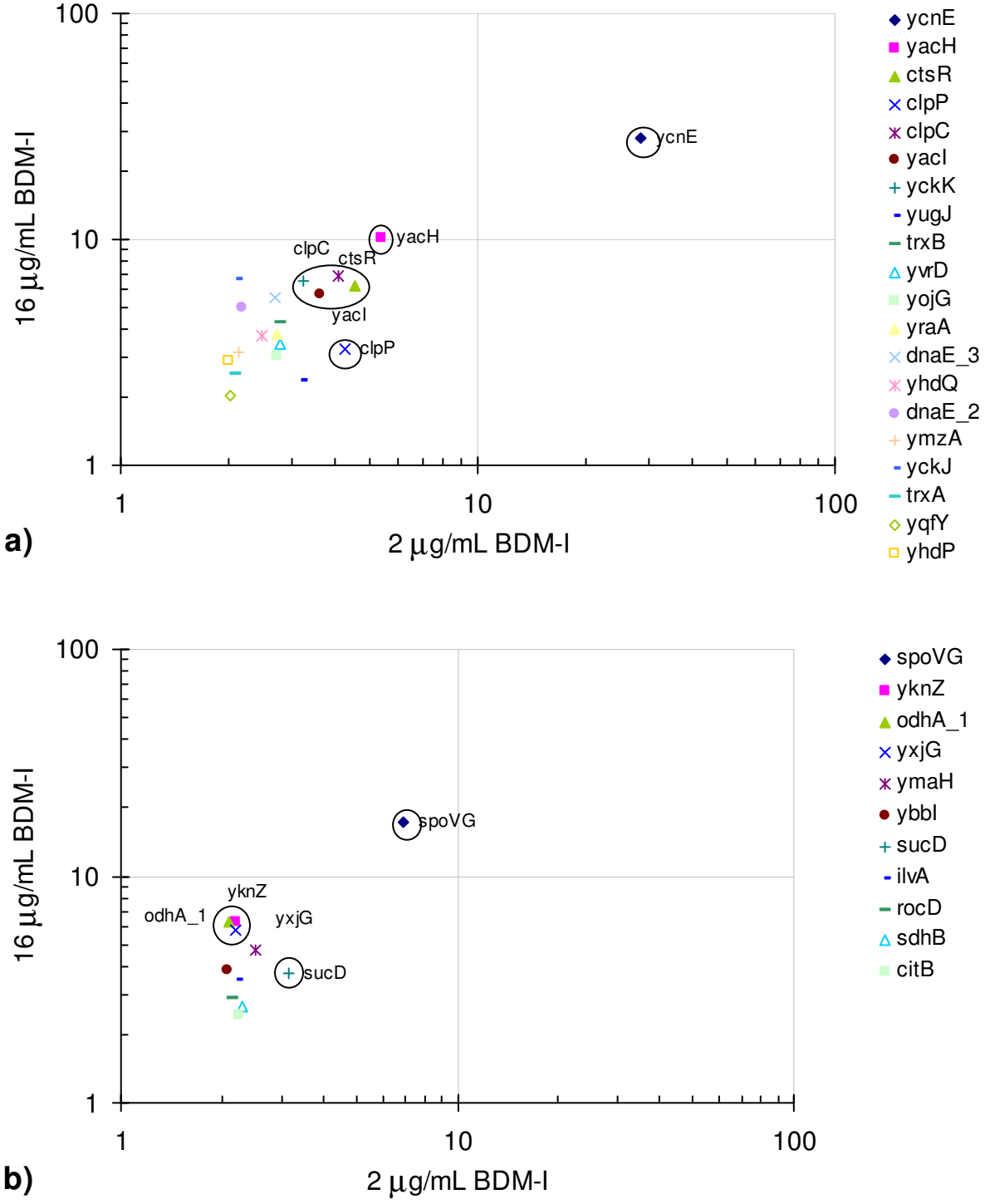


Figure 6-5. Scatter graph showing fold changes of genes commonly up-regulated (a) and down-regulated (b) between 2 and 16 µg/mL treatments of BDM-I.

Circled genes exhibited fold changes ≥3 for both treatments

Genes differentially regulated at one treatment concentration only

To highlight the differences in responses between the two concentrations of BDM-I, genes differentially regulated that were unique to one concentration of BDM-I were identified. In total, 14 genes were identified as being uniquely changed in response to 2 µg/mL BDM-I and 327 in response to 16 µg/mL BDM-I. (Table 6-8).

Table 6-8. Number of genes differentially regulated at either 2 µg/mL or 16 µg/mL BDM-I treatments only.

Treatment	2 µg/mL		16 µg/mL	
	Up-regulated	Down-regulated	Up-regulated	Down-regulated
Number of unique genes	8	6	106	231
No: main functions assigned	9	6	111	238
No: genes identified in primary annotations	6	6	68	183

A complete list of differentially regulated genes identified as being unique to one concentration of BDM-I and their main functional categories is in Appendix G.

Treatment with 2 µg/mL BDM-I resulted in a 3.5-fold up-regulation of *ribB*, the riboflavin synthase α subunit in the main functional category of 'Biosynthesis of cofactors, prosthetic groups and carriers'. The remaining genes unique to 2 µg/mL were changed ≤2-fold and are not listed here (Appendix H).

BDM-I treatment with 16 µg/mL resulted in 104 genes being up-regulated ≥3 fold in response to this concentration alone, 37% of which were not found in the primary functional annotations. The fourteen genes up-regulated ≥5 fold are listed in Table 6-9. The most changed gene in response to 16 µg/mL was *mrgA* (18.5-fold) which has no assigned function. In response to 16 µg/mL BDM-I, 233 probe sets were down-regulated ≥3 fold and 41 of these were down-regulated ≥5 fold (Table 6-10). Genes identified in the general stress

response of *B. subtilis* by Price *et al*. (Goh *et al.*, 2002; Hong *et al.*, 2003; Ng *et al.*, 2003) which were changed by BDM-I 16 µg/mL were the up-regulated *ynfC* (6-fold, listed as not found in JCVI), the down-regulated yvyD (8-fold, protein synthesis) and yxjG (6-fold, not found).

Most of the genes identified as unique in response to 16 µg/mL BDM-I were classified as 'Cellular processes', 'Energy metabolism' or 'Protein synthesis' which are the largest classes in *B. subtilis* and are not considered particularly indicative of mechanism (Data not shown). This could be an indication of global stress adaptive responses to an inhibitory concentration which would fit with the treatment concentrations chosen. It is also likely given that a number of genes have been classed as essential for *B. subtilis* survival. The main roles that stand out as being highly represented beyond that expected are 'Biosynthesis of cofactors, prosthetic groups and carriers' or of 'Purines, pyrimidines, nucleosides and nucleotides.

Table 6-9. Up-regulated genes showing ≥5 fold change and unique to 16 µg/mL BDM-I

Probe set ID	Gene Symbol	Fold change	Main role	Sub role	Common Name
BG10864/mrgA_at	*mrgA*	18.5	not found	not found	not found
BG12034/yclN_at	*yclN*	12.3	Transport & binding proteins	Other	similar to ferrichrome ABC transporter (permease)
BG12863/yetG_at	*yetG*	9.6	not found	not found	not found
BG14055/yuzF_at	*yuzF*	9.2	not found	not found	not found
BG12291/yrhB_at	*yrhB*	7.9	Amino acid biosynthesis	Other	similar to cystathionine gamma-synthase
BG14107/yvgY_at	*yvgY*	7.8	Transport and binding proteins	Other	similar to mercuric transport protein
BG14108/yvgZ_at	*yvgZ*	6.5	not found	not found	not found
BG11828/ynfC_at	*ynfC*	6.4	not found	not found	not found
BG14098/yvgP_at	*yvgP*	6.3	Transport and binding proteins	Cations and iron carrying compounds	similar to hypothetical proteins
BG12325/yshA_at	*yshA*	5.5	not found	not found	not found
BG12878/yfhC_at	*yfhC*	5.1	not found	not found	not found
BG10133/yacB_at	*yacB*	5.1	not found	not found	not found
BG10680/mecA_at	*mecA*	5.0	Cellular processes	DNA transformation	negative regulator of genetic competence
BG12612/lmrA_at	*lmrA*	5.0	Regulatory functions	DNA interactions	transcriptional regulator

not found – not listed in the JCVI database

Table 6-10. Down-regulated genes with ≥5 fold change and unique to 16 µg/mL BDM-I

Probe set ID	Gene Symbol	Fold change	Main role	Sub role	Common Name
BG10240/fliF_at	*fliF*	13.2	Cellular processes	Chemotaxis & motility	flagellar basal-body M-ring protein
BG19003/lctE_at	*lctE*	13.0	not found	not found	not found
BG10706/purQ_at	*purQ*	11.7	Purines, pyrimidines, nucleosides and nucleotides	Purine ribonucleotide biosynthesis	phosphoribosylformylglycinamidine synthetase I
BG10704/yexA_at	*yexA*	10.7	not found	not found	not found
BG11062/pgk_at	*pgk*	10.6	Energy metabolism	Glycolysis/gluconeogenesis	phosphoglycerate kinase
BG14170/yxzG_at	*yxzG*	10.2	not found	not found	not found
BG12605/hisS_at	*hisS*	10.0	Protein synthesis	tRNA aminoacylation	histidyl-tRNA synthetase
BG12838/yerL_at	*yerL*	9.7	not found	not found	not found
BG12660/pycA_3_at	*pycA_*	8.4	Energy metabolism	Glycolysis/gluconeogenesis	pyruvate carboxylase
BG10740/yvyD_at	*yvyD*	7.9	Protein synthesis	Translation factors	alternate gene name: yvil similar to ribosomal
BG11846/wprA_at	*wprA*	7.9	Cell envelope	Other	cell wall-associated protein precursor (CWBP23, CWBP52)
BG10276/hbs_at	*hbs*	7.8	DNA metabolism	DNA replication, recombination, & repair; Chromosome-associated proteins	DNA-binding protein HB
BG10707/purF_at	*purF*	7.5	Purines, pyrimidines, nucleosides, & nucleotides	Purine ribonucleotide biosynthesis	amidophosphoribosyltransferase
BG13785/yrbF_at	*yrbF*	7.3	Protein fate	Protein and peptide secretion and trafficking	similar to hypothetical proteins
BG11384/accC_at	*accC*	7.1	Fatty acid & phospholipid metabolism	Biosynthesis	acetyl-CoA carboxylase, biotin carboxylase
BG10897/tpi_at	*tpi*	7.1	Energy metabolism	Glycolysis/gluconeogenesis	triose phosphate isomerase
BG11023/yvcE_at	*yvcE*	6.9	Cell envelope	Biosynthesis & degradation of murein sacculus & peptidoglycan	alternate gene name: yzkA; similar to cell wall-binding protein
BG11023/yvcE_at	*yvcE*	6.9	not found	not found	not found
BG11846/wprA_1_at	*wprA*	6.9	Cell envelope	Other	cell wall-associated protease precursor
BG10703/purC_at	*purC*	6.6	Purines, pyrimidines, nucleosides, & nucleotides	Purine ribonucleotide biosynthesis	phosphoribosylaminoimidazole-succinocarboxamide synthase
BG12572/aspS_at	*aspS*	6.6	Protein synthesis	tRNA aminoacylation	aspartyl-tRNA synthetase
BG13245/yknY_at	*yknY*	6.6	Transport & binding proteins	Other	similar to ABC transporter (ATP-binding protein)
BG11247/tkt_at	*tkt*	6.5	Energy metabolism	Pentose phosphate pathway	transketolase
BG11135/yxiF_at	*yxiF*	6.3	not found	not found	not found
BG10705/purL_at	*purL*	6.2	Purines, pyrimidines, nucleosides, & nucleotides	Other	phosphoribosylformylglycinamidine synthetase II
BG11816/yktA_at	*yktA*	6.2	not found	not found	not found
BG11460/efp_at	*efp*	6.1	Protein synthesis	Translation factors	translation elongation factor P

BG13236/ykgB_at	*ykgB*	6.1	not found	not found	not found
BG10815/atpB_at	*atpB*	6.0	Energy metabolism	ATP-proton motive force interconversion	ATP synthase (subunit a)
BG13395/yloU_at	*yloU*	6.0	Cellular processes	Adaptations to atypical conditions	similar to alkaline-shock protein
BG10816/atpE_at	*atpE*	6.0	Energy metabolism	ATP-proton motive force interconversion	ATP synthase (subunit c)
BG10622/rocA_at	*rocA*	5.8	Energy metabolism	Amino acids and amines	pyrroline-5 carboxylate dehydrogenase
BG11138/yxiI_at	*yxiI*	5.6	not found	not found	not found
BG10817/atpF_at	*atpF*	5.5	Energy metabolism	ATP-proton motive force interconversion	ATP synthase F0, B subunit
BG13392/yloQ_at	*yloQ*	5.5	not found	not found	not found
BG12839/yerM_at	*yerM*	5.5	Protein synthesis	tRNA aminoacylation	alternate gene name: yedB similar to amidase
BG11709/yqhY_at	*yqhY*	5.5	not found	not found	not found
BG13152/yjbW_at	*yjbW*	5.3	Fatty acid & phospholipid metabolism	Biosynthesis	similar to enoyl- acyl-carrier protein reductase
BG10241/fliG_at	*fliG*	5.3	Cellular processes	Chemotaxis & motility	flagellar motor switch protein
BG12366/pgi_at	*pgi*	5.3	Energy metabolism	Glycolysis/gluconeogenesis	glucose-6-phosphate isomerase
BG13934/ytwP_at	*ytwP*	5.1	Energy metabolism	Sugars	unknown
BG11708/yqhT_at	*yqhT*	5.0	Protein fate	Degradation of proteins, peptides, & glycopeptides	similar to Xaa-Pro dipeptidase

not found – not listed in the JCVI database

6.3.5 Validation of microarray data by quantitative PCR

Relative transcript amounts for selected genes to verify transcriptional expression values were determined by qPCR of cDNA (Section 6.2.5). Uniform expression was seen for *mbl* across untreated and BDM-I treated cells (Figure 6-6). This unchanged mbl gene was included as an internal control. The target genes exhibited differential expression under the two treatment conditions. The class III stress-response ATPase, *clpC,* and the DNA polymerase III subunit α, *dnaE*, were up-regulated while *spoVG*, involved in spore cortex formation, was down-regulated compared to the control and relative to the housekeeping gene *mbl*.

Results from qPCR assays mirrored microarray fold-changes in gene expression (Figure 6-6). All qPCR results for gene expression were within two-fold of results from the microarray for *B. subtilis* treated with 2 µg/mL BDM-I. For *B. subtilis* treated with 16 µg/mL BDM-I, changes detected by qPCR were 4, 5 and 12-fold greater for *dnaE*, *clpC* and *spoVG* respectively, than those calculated from the microarray data. These results highlight the greater sensitivity of the qPCR procedure over microarray transcription analysis for three out of the four genes used here.

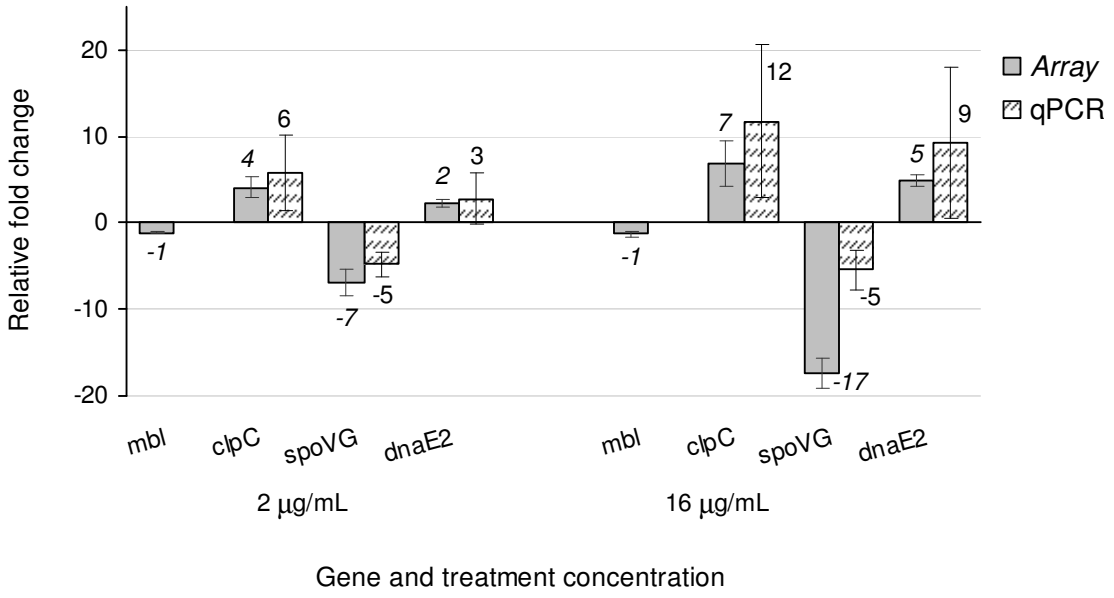


Figure 6-6. Relative fold changes in *B. subtilis* genes compared between Genechip® microarray and qPCR results.

Error bars represent standard deviation. Array fold changes shown in italics, qPCR fold changes in normal text. *mbl* was used as the internal control and baseline comparison (no change). Genes were chosen based on differential regulation in both 2 and 16 µg/mL treatment concentrations.

6.4 DISCUSSION

This preliminary analysis of the *B. subtilis* genome has illustrated the rapid and complex genomic response of bacteria to chemical stress. Using one treatment time and two concentrations, this study has identified that the optimum treatment concentration for further analysis of transcripts in response to BDM-I is likely between 2 and 16 µg/mL. An additional earlier time point, less than the 30 min exposure used here, might help identify time-related specific patterns of expression that could be linked to more directed studies of the genes affected by BDM-I. The percentages of transcribed genes which were significantly changed at 30 min for 2 and 16 µg/mL treatments were 1% and 8%. Similar levels of changed genes have been reported in studies of the transcriptional profiles of other bacteria subjected to antimicrobial agents (2002). The significantly changed genes

covered many functional categories, particularly at the inhibitory concentration of 16 μg/mL, illustrating the complexity of the bacterial response to chemical stress.

The analysis identified genes that were changed at both sub-inhibitory and inhibitory concentrations, most showing a dose response in expression fold change with concentration. Changed genes common to both treatment concentration may include genes specifically altered by BDM-I or genes changed as a consequence of these. The commonly regulated genes (Table 6-6 & Table 6-7) included two greatly up-regulated genes, *ycnE* (28-fold) and *yacH* (10-fold), both of unknown function, which warrant further investigation. Other genes with high fold changes of interest include those relating to 'DNA metabolism', 'purine, pyrimidine, nucleoside and nucleotide synthesis', 'fatty acid biosytnhesis and phospholipid metabolism', which are target areas of antimicrobial compounds not yet investigated for BDM-I. A gene involved in toxin production and toxin resistance ('cellular processes'), *yacI* (6-fold) would also be of interest given the demonstrated inhibition by BDM-I of secreted secondary metabolites (Chapter 4). A gene, *fliF*, in the function of 'cellular processes' and associated with chemotaxis and motility was down-regulated 13-fold in response to only 16 μg/mL BDM-I. This warrants further investigation given its connection with cell signalling and possible links to BDM-I activity. Also of interest for further work is the 7-fold down-regulation of *accC* which is involved in fatty acid biosynthesis, a current antimicrobial target.

A gene significant in endospore formation, *spoVG* was down-regulated 7-fold at 2 μg/mL and 17-fold at 16 μg/mL (Table 6-7). Eymann *et al*., (2000) have identified *spoVG* as part of the stringent response to amino acid and carbon starvation in *B. subtilis*. SpoVG is a protein inhibitor of sporulation involved in spore cortex formation in the stationary phase of sporulation. Two other genes involved in endospore formation were changed at16 μg/mL. A transcriptional regulator of spore proteins, *soj,* was up-regulated

2-fold and *yrbC* (spore coat protein synthesis) was up-regulated 3-fold. Quisel & Grossman (Quisel & Grossman, 2000) showed that Soj, a chromosome partitioning protein with putative ATPase activity, negatively regulates transcription of *spoII* genes and of *spo0A* which itself activates transcription of the *spoII* genes (2004a). The down-regulation of *spoVG* and the up-regulation of *soj* in *Bacillus spp.* will require further investigation to reconcile observations in Chapter 3 of a decrease in endospore formation in *B. cereus* treated with BDM-I. BDM-I may interfere with the complex of signals governing control of spore formation.

Hutter *et al.* (Bandow *et al.*, 2002) identified 12 functionally annotated marker genes suitable for identifying classes of compounds as listed below.

Gene	**Mechanism and/or compound class**	**Reference antibiotic**
fabHB, glpD	Fatty acid synthesis	Cerulenin
yrzl, expZ	Protein synthesis	Clindamycin
ypbG, ytrA, ywoB	Cell wall synthesis (glycopeptides)	Vancomycin
dinB, yneA, yorB	DNA replication (quinolones)	Ciprofloxacin
ydeK	Cell wall synthesis	Cycloserine
yvgS	Transcription	Rifampicin

None of these genes were differentially regulated by BDM-I in this analysis. This suggests that BDM-I does not act on these current major cellular targets for antibiotics. This investigation failed to show an effect of BDM-I on protein or cell wall synthesis or DNA transcription. This supports the liklihood that BDM-I is not acting on a cellular mechanism targeted by the above classes of compounds.

Two genes differentially regulated by both concentrations and identified as essential, *clpC* and *clpP* (protein fate) are part of the σ^B-dependent general stress response (Table 6-6). They have been identified as part of *B. subtilis* response to Rifampin, which inhibits DNA-dependent RNA polymerase and therefore RNA synthesis (2001). A further 2 genes involved in the general stress responses in *B. subtilis* , *yxjG* (σ^B) and *sucD* (ComK

competence transcription factor) were among the most down-regulated genes. The general stress response brings about widespread changes in metabolism in anticipation of stress and is governed by the σ^B transcription factor. Price *et al.* (2002) identified 127 genes in *B. subtilis* as candidates for the σ^B regulon including *yxjG* (methionine synthase II, cobalamine independent). Competence is a cellular state allowing exogenous DNA to be internalised. The competence transcription factor, ComK, is a regulator of the competence response, activating transcription of genes encoding DNA transport proteins. Berka *et al.* (Berka *et al.*, 2002) identified 165 genes that were up-regulated by ComK, including 28 annotated as regulators of transcription or translation (Bandow *et al.*, 2002). This investigation found no inhibition by BDM-I of transcription or translation using a cell-free protein synthesis assay (Chapter 5). Com K is repressed by exposure to rifampin, a RNA polymerase inhibitor (2002). Berka *et al.* (2001) further suggest that the ComK regulon defines an adaptation to stress distinct from sporulation which enables the cell to repair DNA damage, acquire exogenous genes by transformation and use novel substrates. Other genes identified in the general stress response by Price *et al.* (Musumeci *et al.*, 2005) which were changed by BDM-I 16 μg/mL were the down-regulated *yvyD* (8-fold) and the up-regulated *ynfC* (6-fold). Inhibition by BDM-I of genes in the general stress response may act to reduce the resistance of *B. subtilis* to environmental stresses, including the acquisition of antibiotic resistance genes.

B. subtilis stress resistance responses can be tied to phosphatases *yfkJ* and *ywlE* (Mijakovic *et al.*, 2003; Mijakovic *et al.*, 2005a; Musumeci *et al.*, 2005). The *B. subtilis* genome shows orthologs for many phosphatases including four identified LMWPTP's – *yfkJ, arsC (yqcM)*, *ywqE*, *ywlE*. These are involved in protein degradation, detoxification, adaptations to atypical conditions and general stress responses (Bandow *et al.*, 2003; Freiberg *et al.*, 2004a; Gmuender *et al.*, 2001; Goh *et al.*, 2002). Unfortunately, no

annotated phosphatase genes were identified as directly changed by BDM-I from this study.

Not all cellular processes, however, are controlled at the level of gene transcription and detected by transcript levels. Other regulatory mechanisms act at the level of mRNA translation. Post-transcriptional and post-translational modifications such as phosphorylation of proteins, will not be detected by mRNA transcript levels. Proteome-based expression analysis can be used to detect such changes and will complement transcriptional analyses (Freiberg *et al.*, 2004a). Proteomic studies are notoriously labour intensive and focus on a limited set of cytoplasmic proteins with limited molecular weights and isoelectric points (2004a). Proteomic analysis was not attempted in this investigation because of difficulties in detecting low abundance phosphorylated proteins and time consraints and delayed access to highly used laboratory resources. Phosphoproteome searches using ^{32}P and two-dimensional SDS-PAGE are a challenge in bacteria that contain low levels of total phosphotyrosine. Use of Δptp2 (phosphatase deficient strains) can help sensitivity in these investigations. Once a specific phosphatase target is suspected, proteomic studies utilising deficient strains would complement genomic studies and provide more leads for detailing the MOA of BDM-I.

CHAPTER 7 CONCLUSONS AND FUTURE DIRECTIONS

A new mechanism of action

The results of this research support the proposal that 3,4-methylenedioxy-β-nitropropene (BDM-I) acts as a tyrosine mimetic to inhibit bacterial tyrosine phosphatases, the nitropropene side-chain being the pharmacophore. This is a plausible explanation for its broad activity against bacteria, fungi and protozoa and for certain species-specific effects in bacteria. The hypothesis is supported indirectly by the studies reported here. It has not yet been demonstrated that BDM-I directly inhibits any bacterial PTP's in target microbial cells or that such inhibition is responsible for its antimicrobial effects.

Protein tyrosine phosphatases as antimicrobial drug targets

The catalytic site of PTP's is highly conserved across prokaryotic and eukaryotic cells. It is thus expected that the pharmacophore of a PTP inhibitor would act on all PTP's to varying degrees. Tyrosine phosphatases and dual specific phosphatases are promising drug targets for human therapeutics especially as anti-tumour drugs. This work suggests they are also promising targets for anti-infective drugs. Microorganisms, particularly prokaryotes, are evolutionarily removed from mammals and may have adapted phosphatases for different functions, providing selective targets for drug action. A single molecule inhibiting phosphatases can inhibit a family of essential proteins that have multiple cellular functions. Given the multiple mutations that would be required to produce a resistant phenotype, it is unlikely that resistance in pathogens will readily emerge.

Evidence in support of the proposal

BDM-I inhibits both a human and a bacterial PTP in an enzyme assay (Section 5.4.4). Park & Pei (2004) have shown how an unsubstituted benzyl nitroethene with a

similar pharmacophore interacts at the catalytic site (Section 5.4.1). The highly conserved nature of the catalytic site strongly supports the likelihood that BDM-I would inhibit other microbial PTP's in similar enzyme assays and in whole cells. No evidence was found to support action of BDM-I on an already known bacterial target, although not all were investigated in this project. The biological effects of BDM-I, extended in these investigations and presented below, were examined for consistency with this hypothesis.

The cell wall and cytoplasmic membrane of microorganisms are major targets for biocides and many therapeutic antimicrobial agents. BDM-I has been shown by cell and enzyme assays and studies on ultramorphology not to degrade or damage the cell wall/envelope of bacteria or damage the plasma membrane (Chapter 3). No ultra-structural changes in bacterial or yeast cells were observed by TEM. The lipopolysaccharide outer leaflet of Gram negative bacteria is shown not to be a major contributor to the lack of activity against many enteric Gram negative rods (Section 2.3.4).

The intracellular site of action of BDM-I and its ability to act on intracellular pathogens has been extended by demonstration of inhibition of the early stage of the replication cycle of *Chlamydia trachomatis* in mammalian cells. It is proposed that BDM-I inhibits binding or endocytosis of the elementary bodies and formation of vacuoles, but does not interfere with replication of reticulate bodies. Host cell tyrosine phosphatases have been shown to be involved in chlamydial vacuole formation and a probable explanation of BDM-I activity could be inhibition of host PTP's such as cortactin, a phosphotyrosine actin-binding protein associated with vacuole formation (Section 2.5.4).

BDM-I has been shown to be variably and slowly bactericidal to different species. The killing rate is not affected by the growth rate and is time-dependent rather than concentration-dependent. This differentiates BDM-I from antibiotics targeting pathways important to cell replication, namely protein synthesis, cell wall synthesis and

DNA replication, which have their greatest effects on growing cells (Section 2.6.4). BDM-I also did not show synergistic or antagonistic interaction with antibiotics affecting the same pathways, which further supports the inference that the major action of BDM-I lies elsewhere (Section 2.7.4). In a cell-free protein synthesis assay (*E. coli*), BDM-I inhibited neither transcription nor translation of exogenous DNA, supporting other observations that it does not affect ribosomal function (Section 5.3.4).

In contrast, killing of *C. albicans* was rapid and dose-dependent. The different effects in bacterial and yeast cells can be explained by a difference in the significance of PTP's in bacteria and fungi. In bacteria, PTP's have heterogeneous distribution and varied functions which, to date, have not been shown to relate to cell growth or replication. In eukaryotes, PTP's are directly involved in cell growth and the cell cycle and a more uniform and direct effect on yeast replication would be expected (Section 2.6.4). BDM-I shows high and uniformly fungicidal activity against all fungi tested to date.

Thiol-containing compounds were shown to lower BDM-I activity against bacteria. Thiols have been shown to reduce the double bond of the nitropropene side chain, the proposed pharmacophore of BDM-I, inactivating its inhibitory effect on PTP's (Section 2.4.4).

BDM-I is not known to induce or select for resistant phenotypes *in vitro* in species which have readily developed resistance to other agents. It did produce small colony variants in *S. aureus* but these were not stable (Section 4.2.4). The failure of BDM-I to induce resistance is understandable if it acts on a basic and widespread cell signalling mechanism.

Phosphatases in bacteria have been shown to be related to virulence and stress survival mechanisms. Inhibition of virulence factors in addition to inhibition of growth is a desirable attribute in an anti-infective drug for inhibiting colonisation or entry and

limiting microbial pathology. This study has shown that BDM-I inhibits the virulence mechanism of group swarming motility in *Proteus* (Section 4.4.4), though it does not affect individual cell flagellar motility. The former has been shown to be affected by QS signalling. Tyrosine phosphatases have not to date been shown to be involved in QS in bacterial motility.

Protein tyrosine phosphatases have been implicated in the production of exopolysaccharides in bacteria which are virulence factors for many species. A preliminary investigation of the effect of BDM-I on capsule production by *Klebsiella* showed no obvious reduction of capsule size (Section 4.5.4). As a more direct measure of inhibition, BDM-I will be investigated for effect on colonic acid synthesis in *E. coli* in which PTP's have been shown to be involved. BDM-I also inhibited the production of prodigiosin, a pigmented antimicrobial secondary metabolite of *Serratia* (Section 4.3.4). Prodigiosins have been shown to inhibit PTP's in *Streptomyces.* Inhibition of a homologous PTP could explain the inhibitory effect in *Serratia.*

Another stress response and survival mechanism, endospore production, was inhibited by BDM-I as noted in TEM ultrastructure studies of *B. cereus* (Section 3.4.4). Down regulation of *spoVG* and up regulation of *soj*, two genes involved in endospore formation in *Bacillus spp.* were noted in the DNA microarray analysis (Section 6.4). These observations will require further investigation.

BDM-I appears not to interfere with energy metabolism. It did not inhibit ATP production under conditions promoting oxidative energy metabolism which suggests it does not affect either ATP synthase or components of the electron transport system (Section 5.2.4). This is consistent with earlier observations that BDM-I was equally effective against a wide range of facultatively anaerobic species under aerobic or anaerobic conditions.

The pilot whole genome DNA microanalysis has provided several genes of interest for further analysis. No tyrosine phosphatase genes were differentially expressed. None of the marker genes identified by Hutter *et al*. for identifying inhibitors of cell wall synthesis, fatty acid synthesis, protein synthesis or DNA replication, were differentially regulated, further supporting the claim that BDM-I has a novel MOA. Two changed genes related to the stress response of endospore production are mentioned above. A protein involved in the general stress response ComK was also implicated by differential regulation of *sucD*. It has been proposed that this regulates DNA repair and acquisition of exogenous genes by transformation. Inhibition of stress response genes may reduce resistance to environmental stresses, including the acquisition of resistance genes.

Future work

At present little is known of the role of PTP's in bacteria. Identification of more endogenous phosphorylated proteins and their functions will clarify the significance of tyrosine phosphorylation in bacteria and provide specific targets for direct investigation to confirm the action of BDM-I on bacterial PTP's. Further genetic and proteomic analyses to follow up on highly deregulated genes will be undertaken. Some specific investigations are outlined in the chapter discussions.

BDM-I as an anti-infective

BDM-I is microbicidal to many significant bacterial, fungal and protozoal pathogens at concentrations sufficiently selectively toxic to be developed as a topical or mucosal anti-infective drug for human or animal use. A phosphatase inhibitor is likely to have non-specific effects in eukaryotic, especially mammalian cells, because of overlaps with human targets. However, many clinically successful antibiotics are active against targets that are highly conserved but nevertheless show sufficient diversity to be selective targets. BDM-I has several desirable features to support therapeutic use.

There is a renewed need for broad spectrum agents. As drug delivery methods improve, localised treatment of mucosal infections becomes a possibility. This would permit the use of broad spectrum agents that may be too toxic when given systemically, reserving the use of less toxic drugs for the treatment of serious systemic infections. Many gastro-intestinal and genitourinary infections are treatable at the mucosal surface. Activity against major sexually transmitted pathogens suggests a possible use as a vaginal microbicide. BDM-I is highly active against many bacterial and fungal skin pathogens, particularly the dermatophytic fungi, and might be suitable for topical application.

No strains have developed resistance to BDM-I indicating that it will likely induce a low frequency of resistance in microbial populations. The novel MOA would also preclude cross-resistance with currently available drugs. BDM-I thus has the potential for treating pathogenic strains of bacteria, fungi and protozoa without adding to multi-drug resistance. Its likely suppression of virulence mechanisms in pathogens is also a desirable attribute.

BDM-I is amenable to structural modification to improve activity and reduce toxicity. Many analogues have been synthesised and, directed by knowledge of the MOA, further analogues are being synthesised.

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APPENDICES

Appendix A. Media and buffers.

Brucella broth with Campylobacter Growth Supplement

Suspended 28 g of Brucella broth (#211088, BBL™, BD Biosciences, CA, USA) dehydrated powder in 1 L of distilled water and mixed thoroughly. Sterilised by autoclaving at 121°C for 15 minutes. To prepare medium for Campylobacter, added Campylobacter Growth Supplement (Oxoid) when cooled to 50°C. Stored at 2-8°C.

Difco™ Synthetic broth AOAC

Suspended 17 g of Dehydrated powder (#235220, BD Biosciences) in 1 L of purified water and mixed thoroughly. Powder dissolved completely by heating with frequent agitation and boiling for 1-2 minutes. Dispensed into medicine bottles. Autoclaved at 121°C for 20 minutes. Stored at 2-8°C. Before inoculating, aseptically added sterile glucose solution (10%) to a final concentration of 1% .

Horse blood agar

Used Columbia agar base (#CM0331, Oxoid, Cambridge, UK). Added 39 g to 1 L distilled water. Boiled to dissolve the medium completely. Sterilised by autoclaving at 121°C for 15 minutes. Cooled to 50°C and added 5% sterile defibrinated horse blood (#SR0050, Oxoid). Poored into sterile petri dishes and allowed to set. Stored at 2-8°C.

Nutrient agar

Suspended 28 g of dehydrated powder (#CM0003, Oxoid) in 1 litre of distilled water. Solution brought to the boil to dissolve completely. Sterilised by autoclaving at 121°C for 15 minutes. Stored at 2-8°C.

Mueller-Hinton broth

Placed 21.0 g of dehydrated powder (#CM0405, Oxoid) in 1 litre of distilled water and mixed to dissolve completely. Sterilised by autoclaving at 121°C for 15 minutes. Stored at 2-8°C.

Performance was monitored regularly using the standard QC organisms *S. aureus* and *P. aeruginosa*. If the broth did not yield the expected MIC values, the volumes of Mg++ and Ca++ solutions were modified until the MIC values approximated those in Table 3 in the NCCLS 'Methods for Dilution Antimicrobial Susceptibility Tests for bacteria that grow Aerobically' (Approved Standard M7-*A5,* 2000).

Sabouraud liquid medium

Dissolved 30 g of dehydrated powder (#CM0147, Oxoid) in 1 litre of distilled water. Mixed well and distribute into bottles for sterilisation by autoclaving at 121°C for 15 minutes. Stored at 2-8°C.

Yeast nitrogen broth

Prepared Difco™ Yeast nitrogen base without amino acids (#291940, BD Biosciences) by dissolving 6.7 g of base, 5 g of glucose and 5-10 mg of the desired amino acid in 100 mL of purified water (with warming to dissolve). Mixed well and filter sterilised (0.2 µM). Stored at 2-8°C.

YNB (no population growth): 6.7 g of base only in 100 mL prified water.

YNB (suboptimal growth): 6.7 g YNB base, 10 mg glucose and 1.7 mg Casamino acids (#223050, Difco™, BD Biosciences, CA, USA).

YNB (optimal growth): 6.7 g YNB base, 0.5 g glucose and 1.7 g Casamino acids.

Luria broth agar (1.5%)

Combined 10 g/L tryptone (#LP0042, Oxoid), 5 g/L yeast extract (#LP0021, Oxoid), 10 g/L NaCl (#LP0005, Oxoid), 1 g/L Glucose (#LP0071, Oxoid), 1 g/L $MgCl_2 \cdot 6H_2O$ (105833, BDH Chemicals) and 15 g/L Agar (#LP0011, Oxoid). Sterilised by autoclaving at 121°C for 15 minutes.

BDM-I was prepared at 100× test concentrations in 100% DMSO and added at a ratio of 1:100 with measured aliquots of molten agar at 50°C. All plates were poured in a laminar flow cabinet and stored at 4°C for no more than 2 weeks before use.

Immunofluorescence washing buffer

PBS/Tween (20x) pH 7.4: Added to 1 L distilled water: 160 g NaCl, 4 g KH_2PO_4, 23 g $NaH_2PO_4 \cdot 2H_2O$, 4 g KCl and 10 mL Tween 20.

Maintencence media

Minimum essential medium with Earle's balanced salts without glutamine, 500 mL (11-050-0500v, Trace Scientific, Victoria, AUS); foetal calf serum 10 mL (CSL Limited Australia); non-essential amino acids, 100×, 5 mL (21-145-0100v, Trace Scientific); L-glutamine, 200 mM (21-125-0100v, Trace Scientific), 5 mL; 40 µg/mL vancomycin (Sigma-Aldrich); 50 µg/mL gentamycin (Sigma-Aldrich); 0.5 µg/mL cyclohexamide (C7698, Sigma-Aldrich) and 4.5 g/L glucose (#8769, Sigma-Aldrich) 5 mL.

Mounting medium

Combined 5 g potassium iodide, 5 g Iodine, 50 mL distilled water and 50 mL glycerol. Filter through qualitative paper. Stored away from light and store for up to 3 months at room temperature.

Phosphate Buffered Saline (PBS)

Dissolved the following in 800 ml MilliQ H_2O: 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4. Adjusted pH to 7.4. Added MilliQ H_2O to 1L. Sterilized to autoclave. Stored at room temperature.

Formaldehyde Agarose (FA) gel electrophoresis of total RNA

FA Gel: Weighed 1.2 g Agarose (Amresco, Solon, Ohio, USA) and made up to 10 mL with 10× FA gel buffer (below). Added 100 mL of DEPC treated water then heated the mixture in a microwave. After cooling to 50°C, add 1.8 mL of 37% formaldehyde. Mixed by swirling before casting on the gel.

10× FA gel buffer: 200 mM 3-[N-Morpholino]propanesulfonic acid (MOPS) (free acid), 50 mM sodium acetate, 10 mM EDTA (pH 7.0).

RNA sample preparation for FA gel electrophoresis: Add 1 volume of 5× loading buffer (below) to 4 volumes of RNA sample (for example 10 µl of loading buffer and 40 µl of RNA) and mix. Incubate for 3–5 min at 65°C, chill on ice and load onto the equilibrated FA gel.

5× RNA loading buffer: 16 µl saturated aqueous bromophenol blue solution, 80 µl 500 mM EDTA (pH 8.0), 720 µl 37% (12.3 M) formaldehyde, 2 ml 100% glycerol, 3.084 ml formamide, 4 ml 10 x FA gel buffer, RNase-free water to 10 mL. Store approximately 3 months at 4°C.

Run at 5-7 V/cm in 1×FA gel running buffer (below).

1× FA gel running buffer: 100 ml 10× FA gel buffer, 20 ml 37% (12.3 M) formaldehyde, 880 ml RNase-free water.

Transmission electron microscopy

Stock 0.1 M Cacodylate buffer pH7.4. (a) 0.2 M $Na(CH_3)_2AsO_2.3H_2O$ (42.8 g/L); **(b)** 0.2 M HCl (i.e. conc. HCl 36-38% = 10 mL + 603 mL dH2O); 25 mL of A plus 1.4 mL B = pH 7.4.

NaAsO buffer 0.2 M (ProSciTech). 4.3 g / 100 mL ddH2O, pH with 0.2 M HCl. Mix 1:1 with water, 2× glutaraldehyde or 2× OsO_4. Add 5 mM $CaCl_2$ to preserve DNA and membrane. Store cacodylate buffer in clearly labelled airtight container in the fume hood at all times. Do not let dry onto glassware.

Gluteraldehyde. 1% (25% aqueous ampoules, ProSciTech). Dilute in buffer in fumehood. Store at 4°C

Osmium tetroxide (2% aqueous, ProSciTech). Dilute in buffer in fumehood. Store in glass only in an airtight container at 4°C.

ONE-STEP PROCURE 812 RESIN - medium hardness

	Volume 1	Volume 2	Volume 3
PROCURE 812	5 mL	10 mL	20 mL
DDSA	4 mL	8 mL	16 mL
NMA	2 mL	4 mL	8 mL
BDMA	0.3 mL	0.6 mL	1.2 mL
Total	**11.3 mL**	**22.6 mL**	**45.2 mL**

Uranyl acetate staining. Make up saturated solution of Uranyl acetate in 50% methanol (% mL MeOH + 5 mL DW) (~ 1 small spatula / 20 mL). Filter before use (discarding first few drops). Recommended to make and filter at least 10 mL, discarding the first 5 mL. Place drops on dental wax in petri dish and place 1 grid per drop section side down on top of each. Note: Include in petri dish filter paper saturated in Uranyl acetate to avoid precipitation. Leave (5) – 20 mins at RT. Rinse by immersing grids through meniscus of DW (10× each solution) at least three times. Blot and allow to air dry before staining with lead citrate.

Lead Citrate staining. Prepare CO_2 free water by autoclaving and sealing tightly immediately on removal from autoclave. Make 10 M NaOH in CO_2 free water. Add 1 small spatula (0.02 g) Lead citrate to 10 mL. CO_2-free water. Add 0.1 mL of 10 M NaOH in CO_2-free water and seal. Shake vigorously to dissolve. Filter before use. Will keep several days.

CO_2-free staining chamber. Dip filter paper in 0.1 M NaOH and place on wax. Add few NaOH pellets and replace lid. Quickly place drops of stain on dental wax and immediately replace lid. Add grids, section side down to top of drops and cover quickly. Stain 30 sec-10 min. Rinse in CO_2-free water. Blot & air dry.

Appendix B. Data from samples used for RNA extractions.

Table B1. OD and viable count data obtained from treated *B. subtilis* cultures used for Microarray experiments.

		Time (min)			
		0	30	0.00	30.00
Treatment	sample i.d.	CFU/mL		OD_{600nm}	
untreated	3a	7.0E+07	2.1E+08	0.305	not determined
2 µg/mL	3b	not determined			not determined
16 µg/mL	3c	not determined			not determined
untreated	6a	1.4E+08	4.6E+08	0.332	0.294
2 µg/mL	6b	not determined	2.4E+08	0.310	0.305
16 µg/mL	6c	not determined	1.9E+08	0.313	0.227
untreated	7a	1.6E+07	7.0E+07	0.302	0.525
2 µg/mL	7b	not determined	6.0E+07	0.329	0.523
16 µg/mL	7c	not determined	3.0E+07	0.305	0.351

Table B2. Quality data for *B. subtilis* RNA used for Microarray experiments.

Treatment	sample i.d.	**OD_{260nm}**	**OD_{280nm}**	**Ratio**	**Estimated Concentration (µg/mL)**	**Est. Yield µg/50uL**
untreated	**A**	0.239	0.139	1.72	956	48
2 µg/mL	**B**	0.166	0.096	1.73	663	33
16 µg/mL	**C**	0.202	0.117	1.72	807	40
untreated	**A**	0.129	0.071	1.82	514	26
2 µg/mL	**B**	0.228	0.125	1.82	914	46
16 µg/mL	**C**	0.184	0.099	1.86	736	37
untreated	**A**	0.135	0.073	1.82	542	27
2 µg/mL	**B**	0.153	0.084	1.83	610	31
16 µg/mL	**C**	0.091	0.050	1.81	364	18

Refer to Supplementary data for accurate Bioanalyser results.

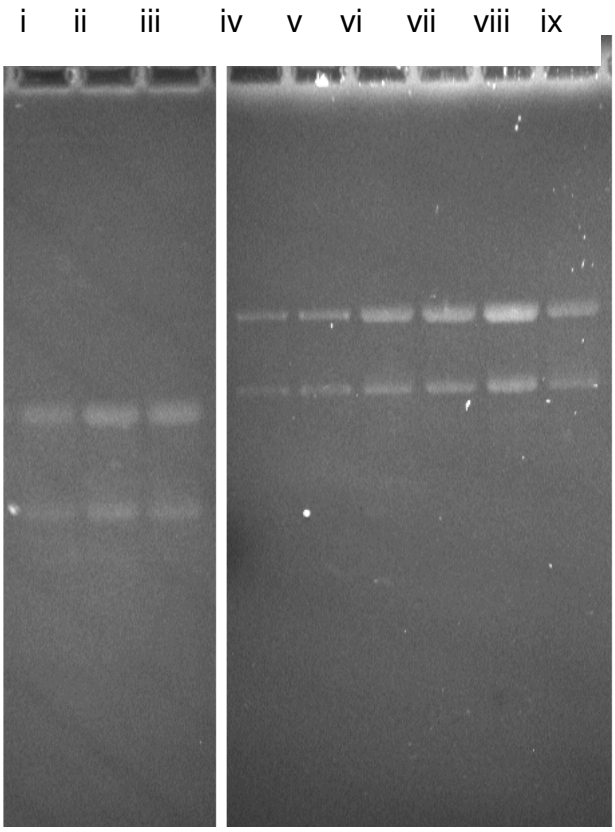


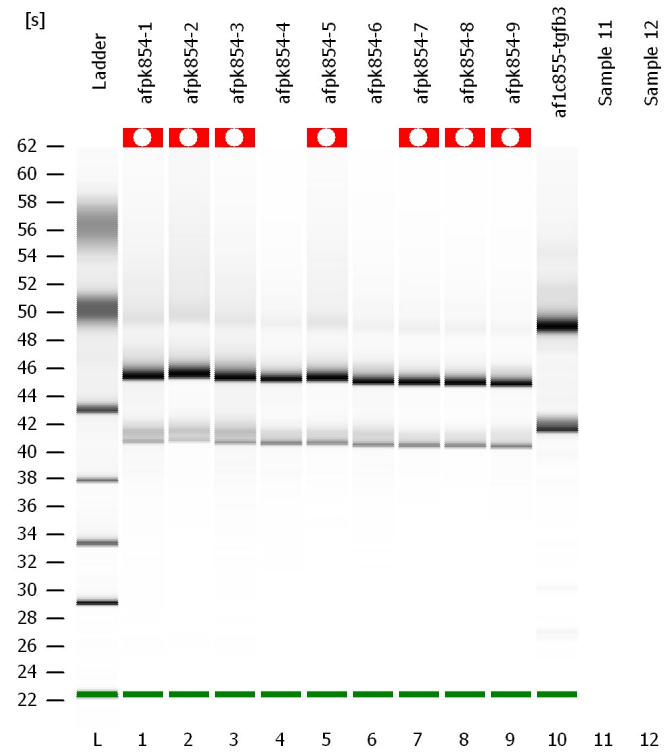
Figure B1. Quality of total RNA isolated from *B. subtilis* ATCC 6633.

Each sample (2 µL) was denatured at 65°C for 5 min and run on a formaldehyde agarose gel. RNA from three replicate experiments is shown. Lanes: i, iv, vii, untreated B. subtilis; ii, v, viii BDM-I 2 µg/mL; iii, vi, ix, BDM-I 2 µg/mL.

Assay Class: EukaryoteTotal RNA Nano
Data Path: D:\XAD files\GEG 1000-1099\1072_2006-11-03_001.xad

Created: 11/3/2006 11:13:34 AM
Modified: 11/3/2006 11:34:49 AM

Electrophoresis File Run Summary



Instrument Information:

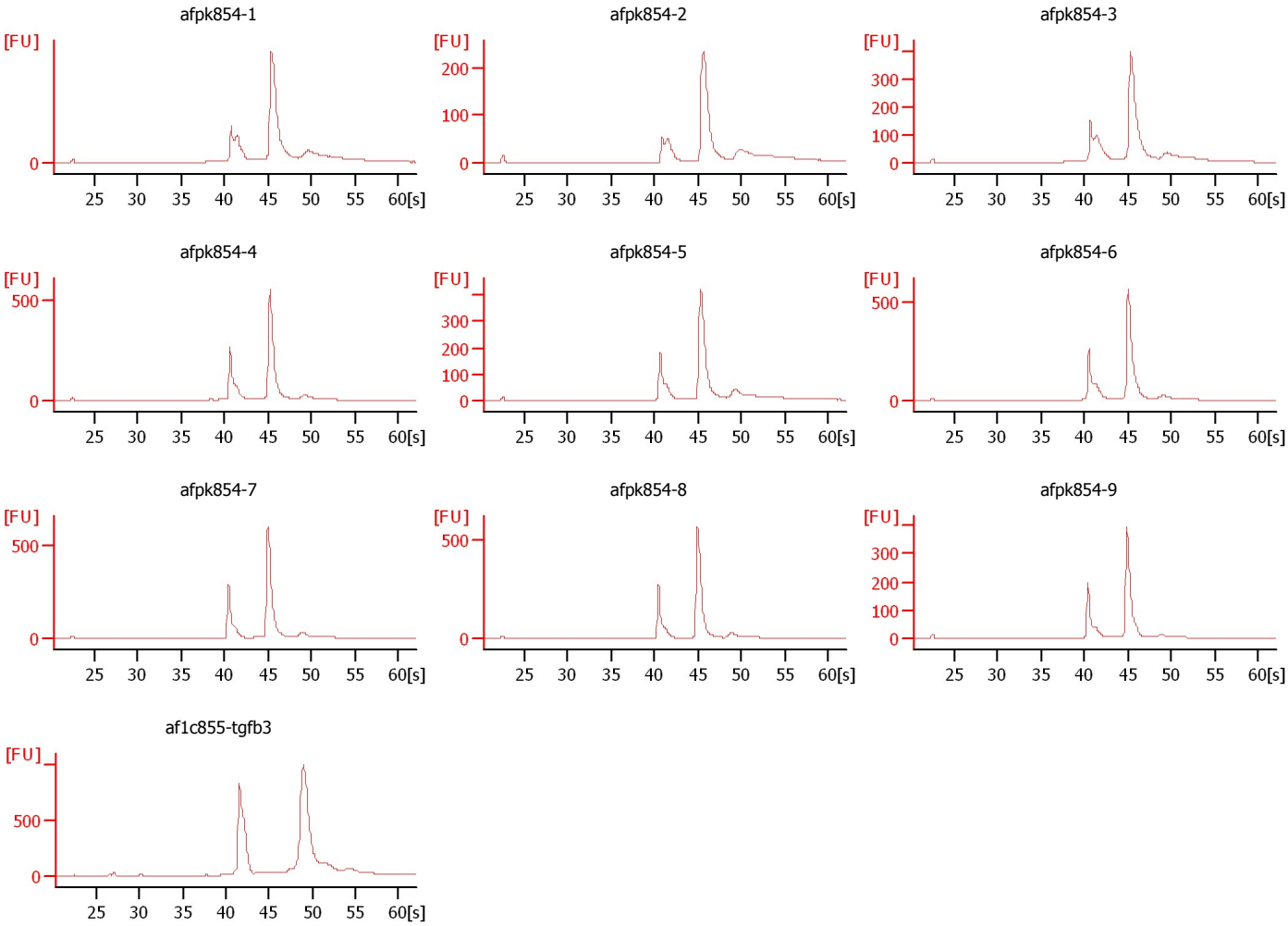
Instrument Name:	DE34903472	Firmware:	C.01.053
Serial#:	DE34903472	Type:	G2938C

Assay Information:

Assay Origin Path:	C:\Program Files\Agilent\2100 bioanalyzer\2100 expert\assays\RNA\Eukaryote Total RNA Nano Series II.xsy
Title:	Eukaryote Total RNA Nano Series II
Version:	2.0
Ladder Concentration:	150.00
Start Time:	19.00
End Time:	69.00
Sizing Accuracy:	15
Quantitation Accuracy:	30
Assay Comments:	Copyright © 2003-2006 Agilent Technologies

Chip Information:

Chip Lot:	
Reagent Kit Lot:	
Chip Comments:	



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Data Path: D:\XAD files\GEG 1000-1099\1072_2006-11-03_001.xad

Created: 11/3/2006 11:13:34 AM
Modified: 11/3/2006 11:34:49 AM

Electrophoresis File Run Summary (Chip Summary)

Sample Name	Sample Comment	Status	Observation	Result Label	Result Color
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afpk854-2		✓			
afpk854-3		✓			
afpk854-4		✓			
afpk854-5		✓			
afpk854-6		✓			
afpk854-7		✓			
afpk854-8		✓			
afpk854-9		✓			
af1c855-tgfb3		✓			
Sample 11					
Sample 12					

Chip Lot # **Reagent Kit Lot #**

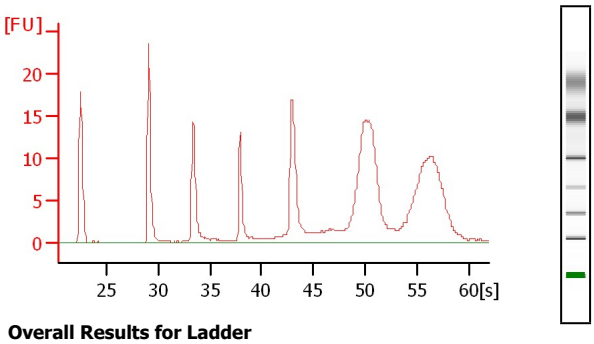
Chip Comments :

Assay Class: EukaryoteTotal RNA Nano
Data Path: D:\XAD files\GEG 1000-1099\1072_2006-11-03_001.xad

Created: 11/3/2006 11:13:34 AM
Modified: 11/3/2006 11:34:49 AM

Electropherogram Summary

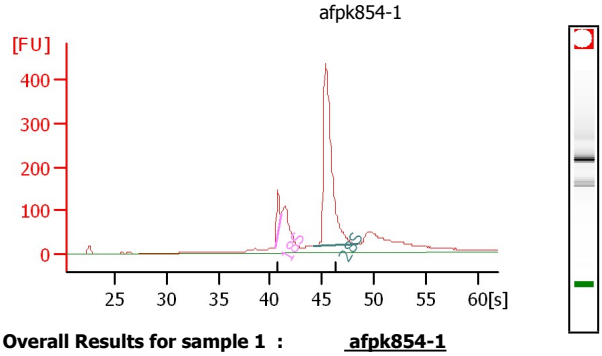
Ladder



Overall Results for Ladder

RNA Area:	234.5
RNA Concentration:	150 ng/µl

afpk854-1



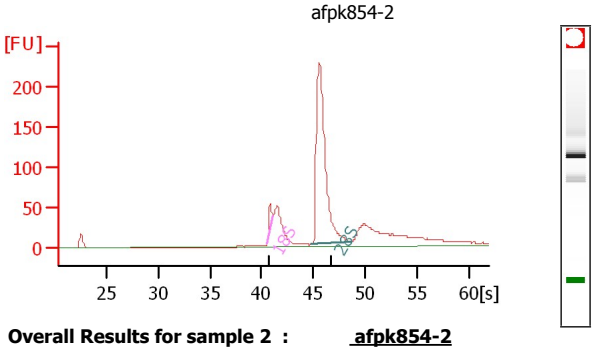
Overall Results for sample 1 : afpk854-1

RNA Area:	1,979.5
RNA Concentration:	1,266 ng/µl
rRNA Ratio [28s / 18s]:	15.2
RNA Integrity Number (RIN):	N/A (B.02.02)

Fragment table for sample 1 : afpk854-1

Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	40.42	41.00	55.2	2.8
28S	44.23	48.42	840.0	42.4

afpk854-2



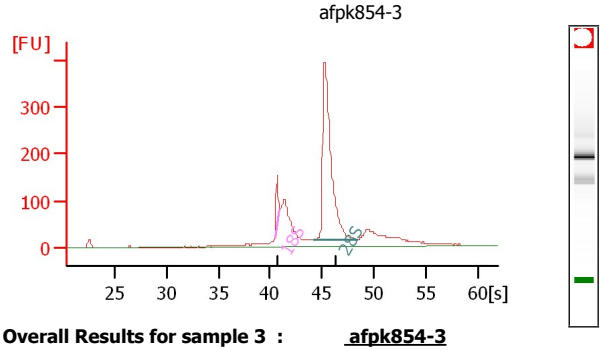
Overall Results for sample 2 : afpk854-2

RNA Area:	948.7
RNA Concentration:	607 ng/µl
rRNA Ratio [28s / 18s]:	23.7
RNA Integrity Number (RIN):	N/A (B.02.02)

Fragment table for sample 2 : afpk854-2

Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	40.54	41.11	20.7	2.2
28S	44.71	48.74	490.2	51.7

afpk854-3



Overall Results for sample 3 : afpk854-3

RNA Area:	1,760.6
RNA Concentration:	1,126 ng/µl
rRNA Ratio [28s / 18s]:	11.6
RNA Integrity Number (RIN):	N/A (B.02.02)

Fragment table for sample 3 : afpk854-3

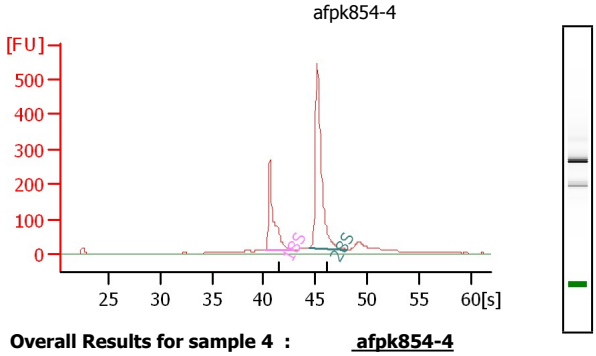
Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	40.41	40.94	66.3	3.8
28S	44.19	48.49	766.0	43.5

Assay Class: EukaryoteTotal RNA Nano
Data Path: D:\XAD files\GEG 1000-1099\1072_2006-11-03_001.xad

Created: 11/3/2006 11:13:34 AM
Modified: 11/3/2006 11:34:49 AM

Electropherogram Summary Continued ...

afpk854-4



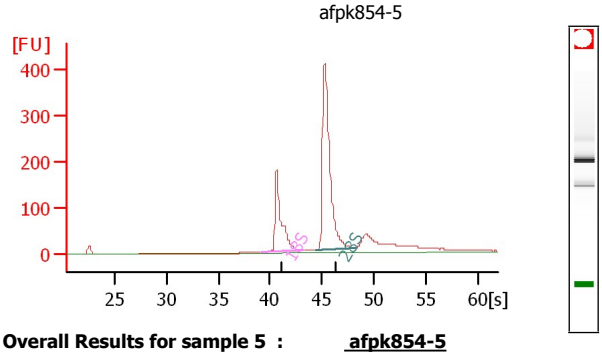
Overall Results for sample 4 : afpk854-4

RNA Area:	1,698.7
RNA Concentration:	1,087 ng/µl
rRNA Ratio [28s / 18s]:	2.1
RNA Integrity Number (RIN):	9.7 (B.02.02)

Fragment table for sample 4 : afpk854-4

Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	40.08	42.90	367.2	21.6
28S	44.47	48.15	780.2	45.9

afpk854-5



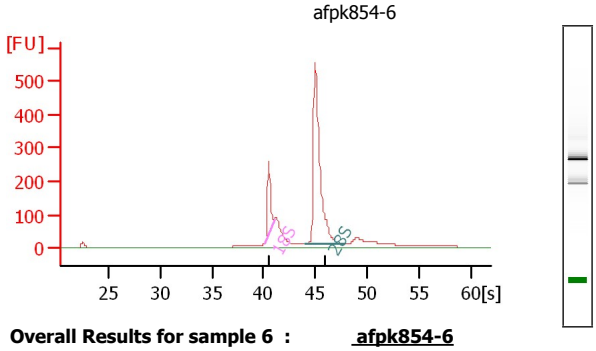
Overall Results for sample 5 : afpk854-5

RNA Area:	1,483.0
RNA Concentration:	949 ng/µl
rRNA Ratio [28s / 18s]:	2.5
RNA Integrity Number (RIN):	N/A (B.02.02)

Fragment table for sample 5 : afpk854-5

Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	39.18	43.13	298.4	20.1
28S	44.42	48.27	738.8	49.8

afpk854-6



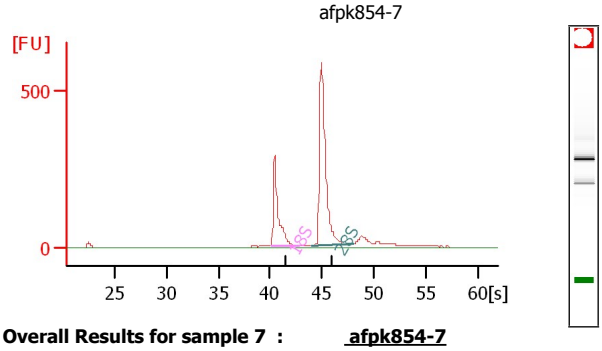
Overall Results for sample 6 : afpk854-6

RNA Area:	1,761.3
RNA Concentration:	1,127 ng/µl
rRNA Ratio [28s / 18s]:	4.8
RNA Integrity Number (RIN):	9.5 (B.02.02)

Fragment table for sample 6 : afpk854-6

Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	40.17	41.04	180.6	10.3
28S	43.99	47.95	873.1	49.6

afpk854-7



Overall Results for sample 7 : afpk854-7

RNA Area:	1,687.2
RNA Concentration:	1,079 ng/µl
rRNA Ratio [28s / 18s]:	2.4
RNA Integrity Number (RIN):	N/A (B.02.02)

Fragment table for sample 7 : afpk854-7

Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	40.09	42.76	374.7	22.2
28S	44.08	48.06	883.5	52.4

Assay Class: EukaryoteTotal RNA Nano
Data Path: D:\XAD files\GEG 1000-1099\1072_2006-11-03_001.xad

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Modified: 11/3/2006 11:34:49 AM

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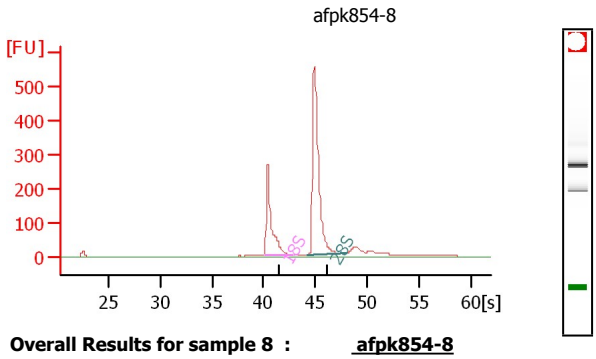
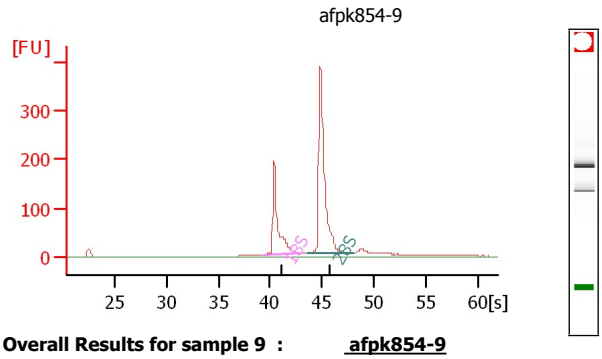


Overall Results for sample 8 : afpk854-8

RNA Area:	1,516.5
RNA Concentration:	970 ng/µl
rRNA Ratio [28s / 18s]:	2.3
RNA Integrity Number (RIN):	N/A (B.02.02)

Fragment table for sample 8 : afpk854-8

Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	40.11	42.85	357.8	23.6
28S	44.17	47.98	835.0	55.1

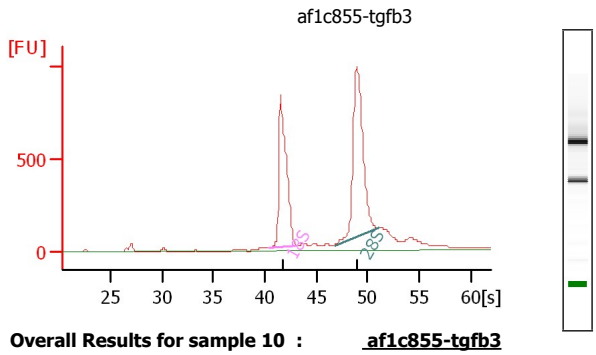


Overall Results for sample 9 : afpk854-9

RNA Area:	1,088.3
RNA Concentration:	696 ng/µl
rRNA Ratio [28s / 18s]:	2.1
RNA Integrity Number (RIN):	N/A (B.02.02)

Fragment table for sample 9 : afpk854-9

Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	39.39	42.78	260.6	23.9
28S	43.67	48.00	558.6	51.3



Overall Results for sample 10 : af1c855-tgfb3

RNA Area:	5,867.1
RNA Concentration:	3,753 ng/µl
rRNA Ratio [28s / 18s]:	1.3
RNA Integrity Number (RIN):	10.0 (B.02.02)

Fragment table for sample 10 : af1c855-tgfb3

Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	40.48	43.21	1,592.0	27.1
28S	46.74	51.11	2,042.2	34.8

Appendix C. GCOS settings.

GCOS 1.0 Expression anlaysis default settings

Default Parameter	Value
Alpha1	0.04
Alpha2	0.06
Tau	0.015
Gamma1L	0.0025
Gamma1H	0.0025
Gamma2L	0.003
Gamma2H	0.003
Perturbation	1.1

Applicable to Genechips with 20 probe pairs/probe set and 28 µm feature size.

Appendix D. GCOS Expression Reports.

Attribute	Value	Value	Value	Value	Value	Value	Value	Value	Value	Value
Barcode	5110260064005304030740198882629	5 7401988826331	7401988826310	7401988826238	7401988826239	7401988826243	7401988826304	7401988826327	7401988826301	
User Created	s9806505	s9806505	s9806505	s9806505	s9806505	s9806505	s9806505	s9806505	s9806505	
User Edited	s9806505	s9806505	s9806505	s9806505	s9806505	s9806505	s9806505	s9806505	s9806505	
Date Created	May 04 2007 03:54PM	May 04 2007 04	May 04 2007 04	May 04 2007 04	May 04 2007 04	May 04 2007 04	May 04 2007 03	May 04 2007 04	May 04 2007 04:04PM	
Date Edited	May 04 2007 03:54PM	May 04 2007 04	May 04 2007 04	May 04 2007 04	May 04 2007 04	May 04 2007 04	May 04 2007 04	May 04 2007 04	May 04 2007 04:04PM	
Archived	NO	NO	NO	NO	NO	NO	NO	NO	NO	
Chip Type	Bsubtilis	Bsubtilis	Bsubtilis	Bsubtilis	Bsubtilis	Bsubtilis	Bsubtilis	Bsubtilis	Bsubtilis	
Assay Type	Expression	Expression	Expression	Expression	Expression	Expression	Expression	Expression	Expression	
NormFactor	1	1	1	1	1	1	1	1	1	
TGT	150	150	150	150	150	150	150	150	150	
ScaleFactor	1.438123	1.845551	0.77579	2.088942	1.643646	1.13954	1.386816	1.640094	1.770404	
Alpha1	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	
Alpha2	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	
Tau	0.015	0.015	0.015	0.015	0.015	0.015	0.015	0.015	0.015	
Gamma1H	0.0025	0.0025	0.0025	0.0025	0.0025	0.0025	0.0025	0.0025	0.0025	
Gamma1L	0.0025	0.0025	0.0025	0.0025	0.0025	0.0025	0.0025	0.0025	0.0025	
Gamma2H	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003	
Gamma2L	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003	
Perturbation	1.1	1.1	1.1	1.1	1.1	1.1	1.1	1.1	1.1	
Algorithm Name	ExpressionStat	ExpressionStat	ExpressionStat	ExpressionStat	ExpressionStat	ExpressionStat	ExpressionStat	ExpressionStat	ExpressionStat	
Baseline File										
Scale Mask File										
Norm Mask File										
Probe Mask File										
NormMethod	User Defined	User Defined	User Defined	User Defined	User Defined	User Defined	User Defined	User Defined	User Defined	
SFMethod	All Probe Sets	All Probe Sets	All Probe Sets	All Probe Sets	All Probe Sets	All Probe Sets	All Probe Sets	All Probe Sets	All Probe Sets	
UsersetUsed	s9806505_BN_Bsubtilis_def	s9806505_BN_I	s9806505_BN_I	s9806505_BN_I	s9806505_BN_I	s9806505_BN_I	s9806505_BN_I	s9806505_BN_I	s9806505_BN_Bsubtilis_de	
Background	Avg:170.77,Stdev:4.11,Max:180.1,Min:159.0	Avg:163.35,Stdev:4.44,Max:174.8,Min:150.6	Avg:156.60,Stdev:3.34,Max:166.6,Min:147.8	Avg:108.63,Stdev:1.98,Max:113.1,Min:102.9	Avg:139.72,Stdev:5.52,Max:154.3,Min:129.5	Avg:147.73,Stdev:3.92,Max:157.9,Min:138.9	Avg:113.52,Stdev:2.27,Max:118.2,Min:107.3	Avg:138.75,Stdev:3.44,Max:146.9,Min:128.6	Avg:95.95,Stdev:2.82,Max:103.2,Min:87.6	
Noise	Avg:17.16,Stdev:0.71,Max:19.1,Min:15.2	Avg:15.81,Stdev:0.83,Max:19.8,Min:14.2	Avg:14.32,Stdev:0.77,Max:16.4,Min:12.6	Avg:9.86,Stdev:0.39,Max:11.0,Min:8.6	Avg:12.67,Stdev:0.67,Max:15.0,Min:11.1	Avg:13.43,Stdev:0.67,Max:15.9,Min:11.9	Avg:10.48,Stdev:0.49,Max:11.8,Min:9.4	Avg:13.68,Stdev:0.55,Max:15.0,Min:12.1	Avg:8.32,Stdev:0.45,Max:9.5,Min:6.9	
RawQ	3.31	3.21	3.09	2.52	2.95	2.99	2.51	2.92	2.27	
Corner+	Avg:443,Count:32	Avg:408,Count:32	Avg:364,Count:32	Avg:257,Count:32	Avg:364,Count:32	Avg:327,Count:32	Avg:297,Count:32	Avg:364,Count:32	Avg:223,Count:32	
Corner-	Avg:15939,Count:32	Avg:16260,Count:32	Avg:15822,Count:32	Avg:16400,Count:32	Avg:15670,Count:32	Avg:15936,Count:32	Avg:16937,Count:32	Avg:15382,Count:32	Avg:17165,Count:32	
Central-	Avg:13853,Count:9	Avg:14085,Count:9	Avg:14039,Count:9	Avg:15395,Count:9	Avg:14649,Count:9	Avg:14635,Count:9	Avg:14771,Count:9	Avg:14317,Count:9	Avg:15232,Count:9	

Report Type: Expression Report
Date: 04:05PM 05/04/2007

Filename: Bs3a.CHP
Probe Array Type: Bsubtilis
Algorithm: Statistical
Probe Pair Thr: 8
Controls: Antisense

Alpha1: 0.04
Alpha2: 0.06
Tau: 0.015
Noise (RawQ): 3.31
Scale Factor (SF): 1.438
TGT Value: 150
Norm Factor (NF): 1

Background:
Avg: 170.7 Std: 4.11 Min: 159.0 Max: 180.10
Noise:
Avg: 17.16 Std: 0.71 Min: 15.20 Max: 19.10
Corner+
Avg: 443 Count: 32
Corner-
Avg: 15939 Count: 32
Central-
Avg: 13853 Count: 9

The following data represents probe sets that exceed the probe pair threshold and are not called "No Call".

Total Probe Sets:	5030	
Number Present:	1450	28.80%
Number Absent:	3459	68.80%
Number Marginal:	121	2.40%

Average Signal (P):	1033.2
Average Signal (A):	33.2
Average Signal (M):	117.8
Average Signal (All):	323.5

Spike Controls:

Probe Set	Sig(5')	Det(5')	Sig(M')	Det(M')	Sig(3')	Det(3')	Sig(all)	Sig(3'/5')
AFFX-BIOB	1137.8	P	1515	P	1373.4	P	1342.05	1.21
AFFX-BIOC	2791.1	P			2167.9	P	2479.5	0.78
AFFX-BIOD	5709.3	P			9224.9	P	7467.09	1.62
AFFX-CRE	19637	P			21663.2	P	20650.12	1.1
AFFX-DAP	163.7	P	562.6	P	263.3	P	329.89	1.61
AFFX-LYS	88.2	A	25.7	A	13.7	A	42.53	0.15
AFFX-PHE	506.8	P	114.4	M	94	A	238.39	0.19
AFFX-THR	123.8	P	136.6	P	113.1	A	124.49	0.91
AFFX-TRP	48	A	74.4	A	15.3	A	45.9	0.32

Report Type: Expression Report
Date: 04:08PM 05/04/2007

Filename: Bs3b.CHP
Probe Array Type: Bsubtilis
Algorithm: Statistical
Probe Pair Thr: 8
Controls: Antisense

Alpha1: 0.04
Alpha2: 0.06
Tau: 0.015
Noise (RawQ): 3.21
Scale Factor (SF): 1.846
TGT Value: 150
Norm Factor (NF): 1

Background:
Avg: 163.3 Std: 4.44 Min: 150.6 Max: 174.80
Noise:
Avg: 15.81 Std: 0.83 Min: 14.20 Max: 19.80
Corner+
Avg: 408 Count: 32
Corner-
Avg: 16260 Count: 32
Central-
Avg: 14085 Count: 9

The following data represents probe sets that exceed the probe pair threshold and are not called "No Call".

Total Probe Sets: 5030
Number Present: 1351 26.90%
Number Absent: 3536 70.30%
Number Marginal: 143 2.80%

Average Signal (P): 1041.5
Average Signal (A): 40.4
Average Signal (M): 140
Average Signal (All): 312.1

Spike Controls:

Probe Set	Sig(5')	Det(5')	Sig(M')	Det(M')	Sig(3')	Det(3')	Sig(all)	Sig(3'/5')
AFFX-BIOB	1320.6	P	1714.9	P	1633.3	P	1556.27	1.24
AFFX-BIOC	3231.3	P			2267.3	P	2749.32	0.7
AFFX-BIOD	6266.8	P			11120.5	P	8693.63	1.77
AFFX-CRE	22780.5	P			26580	P	24680.27	1.17
AFFX-DAP	155.3	P	697.2	P	204.1	P	352.19	1.31
AFFX-LYS	39.7	A	13.6	A	13.4	A	22.24	0.34
AFFX-PHE	420.5	P	69.6	A	57.6	A	182.55	0.14
AFFX-THR	51.1	A	19.7	A	51	A	40.64	1
AFFX-TRP	14.8	A	24	A	10.6	A	16.46	0.72

Report Type: Expression Report
Date: 04:08PM 05/04/2007

Filename: Bs3c.CHP
Probe Array Type: Bsubtilis
Algorithm: Statistical
Probe Pair Thr: 8
Controls: Antisense

Alpha1: 0.04
Alpha2: 0.06
Tau: 0.015
Noise (RawQ): 3.09
Scale Factor (SF): 0.776
TGT Value: 150
Norm Factor (NF): 1

Background:
Avg: 156.6 Std: 3.34 Min: 147.8 Max: 166.60
Noise:
Avg: 14.32 Std: 0.77 Min: 12.60 Max: 16.40
Corner+
Avg: 364 Count: 32
Corner-
Avg: 15822 Count: 32
Central-
Avg: 14039 Count: 9

The following data represents probe sets that exceed the probe pair threshold and are not called "No Call".

Total Probe Sets: 5030
Number Present: 1936 38.50%
Number Absent: 2944 58.50%
Number Marginal: 150 3.00%

Average Signal (P): 717
Average Signal (A): 16.8
Average Signal (M): 54.3
Average Signal (All): 287.4

Spike Controls:

Probe Set	Sig(5')	Det(5')	Sig(M')	Det(M')	Sig(3')	Det(3')	Sig(all)	Sig(3'/5')
AFFX-BIOB	537.4	P	853.5	P	673.8	P	688.23	1.25
AFFX-BIOC	1391.3	P			1059.2	P	1225.24	0.76
AFFX-BIOD	2584.9	P			4207.7	P	3396.26	1.63
AFFX-CRE	9681.7	P			10827	P	10254.34	1.12
AFFX-DAP	92.8	P	149.8	P	85	P	109.22	0.92
AFFX-LYS	60.1	P	33	P	43.5	A	45.55	0.72
AFFX-PHE	244.6	P	23.3	A	19.6	A	95.86	0.08
AFFX-THR	93.4	P	80.7	P	69.1	A	81.09	0.74
AFFX-TRP	9.6	A	30.3	A	3.8	A	14.59	0.4

Report Type: Expression Report
Date: 04:08PM 05/04/2007

Filename: Bs6a.CHP
Probe Array Type: Bsubtilis
Algorithm: Statistical
Probe Pair Thr: 8
Controls: Antisense

Alpha1: 0.04
Alpha2: 0.06
Tau: 0.015
Noise (RawQ): 2.52
Scale Factor (SF): 2.089
TGT Value: 150
Norm Factor (NF): 1

Background:
Avg: 108.6 Std: 1.98 Min: 102.9 Max: 113.10
Noise:
Avg: 9.86 Std: 0.39 Min: 8.60 Max: 11.00
Corner+
Avg: 257 Count: 32
Corner-
Avg: 16400 Count: 32
Central-
Avg: 15395 Count: 9

The following data represents probe sets that exceed the probe pair threshold and are not called "No Call".

Total Probe Sets:	5030	
Number Present:	1542	30.70%
Number Absent:	3326	66.10%
Number Marginal:	162	3.20%

Average Signal (P):	1002.4
Average Signal (A):	30.9
Average Signal (M):	100.8
Average Signal (All):	331

Spike Controls:

Probe Set	Sig(5')	Det(5')	Sig(M')	Det(M')	Sig(3')	Det(3')	Sig(all)	Sig(3'/5')
AFFX-BIOB	1341.3	P	1655.8	P	1787.9	P	1595	1.33
AFFX-BIOC	3341.4	P			2617.6	P	2979.5	0.78
AFFX-BIOD	7564.1	P			12312.8	P	9938.44	1.63
AFFX-CRE	26828.2	P			29288.9	P	28058.53	1.09
AFFX-DAP	251.6	P	613.9	P	248.2	P	371.25	0.99
AFFX-LYS	41.9	A	41.5	A	13.6	A	32.3	0.32
AFFX-PHE	466	P	87.4	P	88	A	213.78	0.19
AFFX-THR	141.3	P	117.1	P	158.3	A	138.91	1.12
AFFX-TRP	26.3	A	140.6	A	13.7	A	60.21	0.52

Report Type: Expression Report
Date: 04:08PM 05/04/2007

Filename: Bs6b.CHP
Probe Array Type: Bsubtilis
Algorithm: Statistical
Probe Pair Thr: 8
Controls: Antisense

Alpha1: 0.04
Alpha2: 0.06
Tau: 0.015
Noise (RawQ): 2.95
Scale Factor (SF): 1.644
TGT Value: 150
Norm Factor (NF): 1

Background:
Avg: 139.7 Std: 5.52 Min: 129.5 Max: 154.30
Noise:
Avg: 12.67 Std: 0.67 Min: 11.10 Max: 15.00
Corner+
Avg: 364 Count: 32
Corner-
Avg: 15670 Count: 32
Central-
Avg: 14649 Count: 9

The following data represents probe sets that exceed the probe pair threshold and are not called "No Call".

Total Probe Sets:	5030	
Number Present:	1579	31.40%
Number Absent:	3305	65.70%
Number Marginal:	146	2.90%

Average Signal (P):	968.4
Average Signal (A):	28.4
Average Signal (M):	105.4
Average Signal (All):	325.7

Spike Controls:

Probe Set	Sig(5')	Det(5')	Sig(M')	Det(M')	Sig(3')	Det(3')	Sig(all)	Sig(3'/5')
AFFX-BIOB	973.4	P	1366.7	P	1268.4	P	1202.86	1.3
AFFX-BIOC	2497.2	P			1944.2	P	2220.71	0.78
AFFX-BIOD	5425.3	P			9410.9	P	7418.12	1.73
AFFX-CRE	19676.3	P			23286	P	21481.15	1.18
AFFX-DAP	206.7	P	890.4	P	260.5	P	452.55	1.26
AFFX-LYS	66.1	A	52.7	A	29.7	A	49.52	0.45
AFFX-PHE	427.8	P	95.5	P	80.6	A	201.32	0.19
AFFX-THR	86	M	40.9	A	89	A	71.97	1.04
AFFX-TRP	10.3	A	21.4	A	14.4	A	15.35	1.4

Report Type: Expression Report
Date: 04:09PM 05/04/2007

Filename: Bs6c.CHP
Probe Array Type: Bsubtilis
Algorithm: Statistical
Probe Pair Thr: 8
Controls: Antisense

Alpha1: 0.04
Alpha2: 0.06
Tau: 0.015
Noise (RawQ): 2.99
Scale Factor (SF): 1.14
TGT Value: 150
Norm Factor (NF): 1

Background:
Avg: 147.7 Std: 3.92 Min: 138.9 Max: 157.90
Noise:
Avg: 13.43 Std: 0.67 Min: 11.90 Max: 15.90
Corner+
Avg: 327 Count: 32
Corner-
Avg: 15936 Count: 32
Central-
Avg: 14635 Count: 9

The following data represents probe sets that exceed the probe pair threshold and are not called "No Call".

Total Probe Sets: 5030
Number Present: 1797 35.70%
Number Absent: 3095 61.50%
Number Marginal: 138 2.70%

Average Signal (P): 839.1
Average Signal (A): 21
Average Signal (M): 68.9
Average Signal (All): 314.6

Spike Controls:

Probe Set	Sig(5')	Det(5')	Sig(M')	Det(M')	Sig(3')	Det(3')	Sig(all)	Sig(3'/5')
AFFX-BIOB	572.2	P	926.9	P	950.2	P	816.44	1.66
AFFX-BIOC	1796.4	P			1413.3	P	1604.87	0.79
AFFX-BIOD	3620.2	P			6565.5	P	5092.84	1.81
AFFX-CRE	13393.2	P			16176.4	P	14784.78	1.21
AFFX-DAP	107.8	P	174.3	P	78.4	P	120.17	0.73
AFFX-LYS	37.6	A	35.2	A	20.6	A	31.11	0.55
AFFX-PHE	247.1	P	24.1	A	14	A	95.07	0.06
AFFX-THR	45	A	56.7	P	101.2	A	67.64	2.25
AFFX-TRP	10	A	21.2	A	5.6	A	12.28	0.55

Report Type: Expression Report
Date: 04:09PM 05/04/2007

Filename: Bs7a.CHP
Probe Array Type: Bsubtilis
Algorithm: Statistical
Probe Pair Thr: 8
Controls: Antisense

Alpha1: 0.04
Alpha2: 0.06
Tau: 0.015
Noise (RawQ): 2.51
Scale Factor (SF): 1.387
TGT Value: 150
Norm Factor (NF): 1

Background:
Avg: 113.5 Std: 2.27 Min: 107.3 Max: 118.20
Noise:
Avg: 10.48 Std: 0.49 Min: 9.40 Max: 11.80
Corner+
Avg: 297 Count: 32
Corner-
Avg: 16937 Count: 32
Central-
Avg: 14771 Count: 9

The following data represents probe sets that exceed the probe pair threshold and are not called "No Call".

Total Probe Sets:	5030	
Number Present:	1773	35.20%
Number Absent:	3109	61.80%
Number Marginal:	148	2.90%

Average Signal (P):	857.9
Average Signal (A):	23
Average Signal (M):	74.4
Average Signal (All):	318.8

Spike Controls:

Probe Set	Sig(5')	Det(5')	Sig(M')	Det(M')	Sig(3')	Det(3')	Sig(all)	Sig(3'/5')
AFFX-BIOB	922.5	P	1295.3	P	1180.2	P	1132.68	1.28
AFFX-BIOC	2350.4	P			1842.9	P	2096.68	0.78
AFFX-BIOD	4818.9	P			7815.1	P	6317.02	1.62
AFFX-CRE	17937.9	P			19053	P	18495.43	1.06
AFFX-DAP	218.1	P	620.5	P	277.1	P	371.93	1.27
AFFX-LYS	58	A	30.2	A	23	A	37.06	0.4
AFFX-PHE	507.9	P	87.1	P	85.6	A	226.88	0.17
AFFX-THR	132.8	P	113.5	P	139.3	A	128.54	1.05
AFFX-TRP	53.6	A	155	M	25.6	A	78.09	0.48

Report Type: Expression Report
Date: 04:09PM 05/04/2007

Filename: Bs7b.CHP
Probe Array Type: Bsubtilis
Algorithm: Statistical
Probe Pair Thr: 8
Controls: Antisense

Alpha1: 0.04
Alpha2: 0.06
Tau: 0.015
Noise (RawQ): 2.92
Scale Factor (SF): 1.64
TGT Value: 150
Norm Factor (NF): 1

Background:
Avg: 138.7 Std: 3.44 Min: 128.6 Max: 146.90
Noise:
Avg: 13.68 Std: 0.55 Min: 12.10 Max: 15.00
Corner+
Avg: 364 Count: 32
Corner-
Avg: 15382 Count: 32
Central-
Avg: 14317 Count: 9

The following data represents probe sets that exceed the probe pair threshold and are not called "No Call".

Total Probe Sets:	5030	
Number Present:	1554	30.90%
Number Absent:	3345	66.50%
Number Marginal:	131	2.60%

Average Signal (P):	1037.1
Average Signal (A):	27
Average Signal (M):	97.3
Average Signal (All):	340.9

Spike Controls:

Probe Set	Sig(5')	Det(5')	Sig(M')	Det(M')	Sig(3')	Det(3')	Sig(all)	Sig(3'/5')
AFFX-BIOB	975.6	P	1472.7	P	1570.5	P	1339.61	1.61
AFFX-BIOC	3128.2	P			2139.8	P	2633.99	0.68
AFFX-BIOD	5671.2	P			10303.7	P	7987.44	1.82
AFFX-CRE	21475.5	P			23685.6	P	22580.56	1.1
AFFX-DAP	235.3	P	806.7	P	261.1	P	434.38	1.11
AFFX-LYS	67.3	A	29.6	A	30	A	42.29	0.45
AFFX-PHE	428.8	P	87.2	P	78	A	198.02	0.18
AFFX-THR	78.1	P	71.8	P	83.4	A	77.77	1.07
AFFX-TRP	9.7	A	16.5	A	13.7	A	13.33	1.41

Report Type: Expression Report
Date: 04:10PM 05/04/2007

Filename: Bs7c.CHP
Probe Array Type: Bsubtilis
Algorithm: Statistical
Probe Pair Thr: 8
Controls: Antisense

Alpha1: 0.04
Alpha2: 0.06
Tau: 0.015
Noise (RawQ): 2.27
Scale Factor (SF): 1.77
TGT Value: 150
Norm Factor (NF): 1

Background:
Avg: 95.95 Std: 2.82 Min: 87.60 Max: 103.20
Noise:
Avg: 8.32 Std: 0.45 Min: 6.90 Max: 9.50
Corner+
Avg: 223 Count: 32
Corner-
Avg: 17165 Count: 32
Central-
Avg: 15232 Count: 9

The following data represents probe sets that exceed the probe pair threshold and are not called "No Call".

Total Probe Sets: 5030
Number Present: 1721 34.20%
Number Absent: 3170 63.00%
Number Marginal: 139 2.80%

Average Signal (P): 973.5
Average Signal (A): 22.4
Average Signal (M): 68.7
Average Signal (All): 349.1

Spike Controls:

Probe Set	Sig(5')	Det(5')	Sig(M')	Det(M')	Sig(3')	Det(3')	Sig(all)	Sig(3'/5')
AFFX-BIOB	794.1	P	1455	P	1422.6	P	1223.89	1.79
AFFX-BIOC	2680.1	P			1842.9	P	2261.47	0.69
AFFX-BIOD	4503.5	P			8424.6	P	6464.05	1.87
AFFX-CRE	17314	P			19968.4	P	18641.17	1.15
AFFX-DAP	95.8	P	143.6	P	81.1	P	106.84	0.85
AFFX-LYS	35.9	A	46.7	A	41	A	41.2	1.14
AFFX-PHE	280.6	P	11	A	16.1	A	102.56	0.06
AFFX-THR	39.5	A	33	A	79.2	A	50.57	2.01
AFFX-TRP	4.8	A	17.4	A	6.2	A	9.45	1.28

Appendix E. GCOS Comparison Reports.

Report Type:	Comparison Report
Date:	04:37PM 05/04/2007

Filename:		3a&3b.CHP
Probe Array Type:	Bsubtilis	
Algorithm:		Statistical
Probe Pair Thr:	8	
Controls:		Antisense

Alpha1:		0.04
Alpha2:		0.06
Tau:		0.015
Noise (RawQ):	3.21	
Scale Factor (SF):	1.846	
TGT Value:		150
Norm Factor (NF):	1	
Baseline filename:	Bs3a.CHP	
Gamma1H:		0.0025
Gamma1L:		0.0025
Gamma2H:		0.003
Gamma2L:		0.003
Perturbation:	1.1	
Baseline Noise (RawQ):	3.31	
Baseline Scale Factor (SF):	1.438123	

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	mean	stdev	min	max
Background:	163.35	4.44	150.6	174.8
Noise:	15.81	0.83	14.2	19.8
			count	
Corner+	408		32	
Corner-	16260		32	
Central-	14085		9	

The following data represents probe sets that exceed the probe pair threshold and are not called "No Call".

Total Probe Sets:	5030	
Number Present:	1351	26.90%
Number Absent:	3536	70.30%
Number Marginal:	143	2.80%
Average Signal (P):	1041.5	
Average Signal (A):	40.4	
Average Signal (M):	140	
Average Signal (All):	312.1	
Number Increase:	297	5.90%
Number Decrease:	317	6.30%
Number MIncrease:	11	0.20%
Number MDecrease:	6	0.10%
Number No Change:	4399	87.50%
Number (A/M->P, MI/I)	42	0.80%
Number (P->A/M, MD/D)	55	1.10%
Number (P->P, MI/I)	196	3.90%
Number (P->P, MD/D)	238	4.70%
Number (A/M->A/M, MI/I)	69	1.40%
Number (A/M->A/M, MD/D)	30	0.60%
Number (P->P, NC)	811	16.10%
Number (A/M->A/M, NC)	3375	67.10%

Number Increase:

(0<=SLR<1):	212
(SLR>=1):	96
(SLR>=2):	19
(SLR>=3):	3
(SLR>=4):	1

Number Decrease:

(0>=SLR>-1):	228
(SLR<=-1):	95
(SLR<=-2):	16
(SLR<=-3):	2
(SLR<=-4):	0

Spike Controls:

Probe Set	Sig(5')	Det(5')	Sig(M')	Det(M')	Sig(3')	Det(3')	Sig(all)	Sig(3'/5')
AFFX-BIOB	1320.6	P	1714.9	P	1633.3	P	1556.27	1.24
AFFX-BIOC	3231.3	P			2267.3	P	2749.32	0.7
AFFX-BIOD	6266.8	P			11120.5	P	8693.63	1.77
AFFX-CRE	22780.5	P			26580	P	24680.27	1.17
AFFX-DAP	155.3	P	697.2	P	204.1	P	352.19	1.31
AFFX-LYS	39.7	A	13.6	A	13.4	A	22.24	0.34
AFFX-PHE	420.5	P	69.6	A	57.6	A	182.55	0.14
AFFX-THR	51.1	A	19.7	A	51	A	40.64	1
AFFX-TRP	14.8	A	24	A	10.6	A	16.46	0.72

Report Type:	Comparison Report
Date:	04:43PM 05/04/2007

Filename:	6a&6b.CHP
Probe Array Type:	Bsubtilis
Algorithm:	Statistical
Probe Pair Thr:	8
Controls:	Antisense

Alpha1:	0.04
Alpha2:	0.06
Tau:	0.015
Noise (RawQ):	2.95
Scale Factor (SF):	1.644
TGT Value:	150
Norm Factor (NF):	1
Baseline filename:	Bs6a.CHP
Gamma1H:	0.0025
Gamma1L:	0.0025
Gamma2H:	0.003
Gamma2L:	0.003
Perturbation:	1.1
Baseline Noise (RawQ):	2.52
Baseline Scale Factor (SF):	2.088942

	mean	stdev	min	max
Background:	139.72	5.52	129.5	154.3
Noise:	12.67	0.67	11.1	15
			count	
Corner+	364		32	
Corner-	15670		32	
Central-	14649		9	

The following data represents probe sets that exceed the probe pair threshold and are not called "No Call".

Total Probe Sets:	5030	
Number Present:	1579	31.40%
Number Absent:	3305	65.70%
Number Marginal:	146	2.90%
Average Signal (P):	968.4	
Average Signal (A):	28.4	
Average Signal (M):	105.4	
Average Signal (All):	325.7	
Number Increase:	308	6.10%
Number Decrease:	279	5.50%
Number MIncrease:	8	0.20%
Number MDecrease:	6	0.10%
Number No Change:	4429	88.10%
Number (A/M->P, MI/I)	43	0.90%
Number (P->A/M, MD/D)	40	0.80%
Number (P->P, MI/I)	228	4.50%
Number (P->P, MD/D)	217	4.30%
Number (A/M->A/M, MI/I)	45	0.90%
Number (A/M->A/M, MD/D)	28	0.60%
Number (P->P, NC)	964	19.20%
Number (A/M->A/M, NC)	3245	64.50%

Number Increase:

(0<=SLR<1):	258
(SLR>=1):	57
(SLR>=2):	9
(SLR>=3):	3
(SLR>=4):	1

Number Decrease:

(0>=SLR>-1):	210
(SLR<=-1):	75
(SLR<=-2):	13
(SLR<=-3):	3
(SLR<=-4):	0

Spike Controls:

Probe Set	Sig(5')	Det(5')	Sig(M')	Det(M')	Sig(3')	Det(3')	Sig(all)	Sig(3'/5')
AFFX-BIOB	973.4	P	1366.7	P	1268.4	P	1202.86	1.3
AFFX-BIOC	2497.2	P			1944.2	P	2220.71	0.78
AFFX-BIOD	5425.3	P			9410.9	P	7418.12	1.73
AFFX-CRE	19676.3	P			23286	P	21481.15	1.18
AFFX-DAP	206.7	P	890.4	P	260.5	P	452.55	1.26
AFFX-LYS	66.1	A	52.7	A	29.7	A	49.52	0.45
AFFX-PHE	427.8	P	95.5	P	80.6	A	201.32	0.19
AFFX-THR	86	M	40.9	A	89	A	71.97	1.04
AFFX-TRP	10.3	A	21.4	A	14.4	A	15.35	1.4

Report Type:	Comparison Report
Date:	04:43PM 05/04/2007

Filename:	7a&7b.CHP
Probe Array Type:	Bsubtilis
Algorithm:	Statistical
Probe Pair Thr:	8
Controls:	Antisense

Alpha1:	0.04
Alpha2:	0.06
Tau:	0.015
Noise (RawQ):	2.92
Scale Factor (SF):	1.64
TGT Value:	150
Norm Factor (NF):	1
Baseline filename:	Bs7a.CHP
Gamma1H:	0.0025
Gamma1L:	0.0025
Gamma2H:	0.003
Gamma2L:	0.003
Perturbation:	1.1
Baseline Noise (RawQ):	2.51
Baseline Scale Factor (SF):	1.386816

	mean	stdev	min	max
Background:	138.75	3.44	128.6	146.9
Noise:	13.68	0.55	12.1	15
			count	
Corner+	364		32	
Corner-	15382		32	
Central-	14317		9	

The following data represents probe sets that exceed the probe pair threshold and are not called "No Call".

Total Probe Sets:	5030	
Number Present:	1554	30.90%
Number Absent:	3345	66.50%
Number Marginal:	131	2.60%
Average Signal (P):	1037.1	
Average Signal (A):	27	
Average Signal (M):	97.3	
Average Signal (All):	340.9	
Number Increase:	274	5.40%
Number Decrease:	353	7.00%
Number MIncrease:	19	0.40%
Number MDecrease:	9	0.20%
Number No Change:	4375	87.00%
Number (A/M->P, MI/I)	31	0.60%
Number (P->A/M, MD/D)	56	1.10%
Number (P->P, MI/I)	153	3.00%
Number (P->P, MD/D)	288	5.70%
Number (A/M->A/M, MI/I)	108	2.10%
Number (A/M->A/M, MD/D)	18	0.40%
Number (P->P, NC)	1040	20.70%
Number (A/M->A/M, NC)	3058	60.80%

Number Increase:

(0<=SLR<1):	197
(SLR>=1):	96
(SLR>=2):	10
(SLR>=3):	3
(SLR>=4):	1

Number Decrease:

(0>=SLR>-1):	305
(SLR<=-1):	54
(SLR<=-2):	11
(SLR<=-3):	2
(SLR<=-4):	0

Spike Controls:

Probe Set	Sig(5')	Det(5')	Sig(M')	Det(M')	Sig(3')	Det(3')	Sig(all)	Sig(3'/5')
AFFX-BIOB	975.6	P	1472.7	P	1570.5	P	1339.61	1.61
AFFX-BIOC	3128.2	P			2139.8	P	2633.99	0.68
AFFX-BIOD	5671.2	P			10303.7	P	7987.44	1.82
AFFX-CRE	21475.5	P			23685.6	P	22580.56	1.1
AFFX-DAP	235.3	P	806.7	P	261.1	P	434.38	1.11
AFFX-LYS	67.3	A	29.6	A	30	A	42.29	0.45
AFFX-PHE	428.8	P	87.2	P	78	A	198.02	0.18
AFFX-THR	78.1	P	71.8	P	83.4	A	77.77	1.07
AFFX-TRP	9.7	A	16.5	A	13.7	A	13.33	1.41

Report Type:	Comparison Report
Date:	04:42PM 05/04/2007

Filename:	3a&3c.CHP
Probe Array Type:	Bsubtilis
Algorithm:	Statistical
Probe Pair Thr:	8
Controls:	Antisense

Alpha1:	0.04
Alpha2:	0.06
Tau:	0.015
Noise (RawQ):	3.09
Scale Factor (SF):	0.776
TGT Value:	150
Norm Factor (NF):	1
Baseline filename:	Bs3a.CHP
Gamma1H:	0.0025
Gamma1L:	0.0025
Gamma2H:	0.003
Gamma2L:	0.003
Perturbation:	1.1
Baseline Noise (RawQ):	3.31
Baseline Scale Factor (SF):	1.438123

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	mean	stdev	min	max
Background:	156.6	3.34	147.8	166.6
Noise:	14.32	0.77	12.6	16.4
			count	
Corner+	364		32	
Corner-	15822		32	
Central-	14039		9	

The following data represents probe sets that exceed the probe pair threshold and are not called "No Call".

Total Probe Sets:	5030	
Number Present:	1936	38.50%
Number Absent:	2944	58.50%
Number Marginal:	150	3.00%
Average Signal (P):	717	
Average Signal (A):	16.8	
Average Signal (M):	54.3	
Average Signal (All):	287.4	
Number Increase:	561	11.20%
Number Decrease:	1171	23.30%
Number MIncrease:	11	0.20%
Number MDecrease:	41	0.80%
Number No Change:	3246	64.50%
Number (A/M->P, MI/I)	184	3.70%
Number (P->A/M, MD/D)	117	2.30%
Number (P->P, MI/I)	372	7.40%
Number (P->P, MD/D)	460	9.10%
Number (A/M->A/M, MI/I)	16	0.30%
Number (A/M->A/M, MD/D)	604	12.00%
Number (P->P, NC)	499	9.90%
Number (A/M->A/M, NC)	2355	46.80%

Number Increase:

(0<=SLR<1):	288
(SLR>=1):	282
(SLR>=2):	108
(SLR>=3):	30
(SLR>=4):	9

Number Decrease:

(0>=SLR>-1):	235
(SLR<=-1):	972
(SLR<=-2):	284
(SLR<=-3):	67
(SLR<=-4):	8

Spike Controls:

Probe Set	Sig(5')	Det(5')	Sig(M')	Det(M')	Sig(3')	Det(3')	Sig(all)	Sig(3'/5')
AFFX-BIOB	537.4	P	853.5	P	673.8	P	688.23	1.25
AFFX-BIOC	1391.3	P			1059.2	P	1225.24	0.76
AFFX-BIOD	2584.9	P			4207.7	P	3396.26	1.63
AFFX-CRE	9681.7	P			10827	P	10254.34	1.12
AFFX-DAP	92.8	P	149.8	P	85	P	109.22	0.92
AFFX-LYS	60.1	P	33	P	43.5	A	45.55	0.72
AFFX-PHE	244.6	P	23.3	A	19.6	A	95.86	0.08
AFFX-THR	93.4	P	80.7	P	69.1	A	81.09	0.74
AFFX-TRP	9.6	A	30.3	A	3.8	A	14.59	0.4

Report Type:	Comparison Report
Date:	04:43PM 05/04/2007

Filename:	6a&6c.CHP
Probe Array Type:	Bsubtilis
Algorithm:	Statistical
Probe Pair Thr:	8
Controls:	Antisense

Alpha1:	0.04
Alpha2:	0.06
Tau:	0.015
Noise (RawQ):	2.99
Scale Factor (SF):	1.14
TGT Value:	150
Norm Factor (NF):	1
Baseline filename:	Bs6a.CHP
Gamma1H:	0.0025
Gamma1L:	0.0025
Gamma2H:	0.003
Gamma2L:	0.003
Perturbation:	1.1
Baseline Noise (RawQ):	2.52
Baseline Scale Factor (SF):	2.088942

	mean	stdev	min	max
Background:	147.73	3.92	138.9	157.9
Noise:	13.43	0.67	11.9	15.9
			count	
Corner+	327		32	
Corner-	15936		32	
Central-	14635		9	

The following data represents probe sets that exceed the probe pair threshold and are not called "No Call".

Total Probe Sets:	5030	
Number Present:	1797	35.70%
Number Absent:	3095	61.50%
Number Marginal:	138	2.70%
Average Signal (P):	839.1	
Average Signal (A):	21	
Average Signal (M):	68.9	
Average Signal (All):	314.6	
Number Increase:	586	11.70%
Number Decrease:	882	17.50%
Number MIncrease:	10	0.20%
Number MDecrease:	18	0.40%
Number No Change:	3534	70.30%
Number (A/M->P, MI/I)	171	3.40%
Number (P->A/M, MD/D)	172	3.40%
Number (P->P, MI/I)	392	7.80%
Number (P->P, MD/D)	457	9.10%
Number (A/M->A/M, MI/I)	33	0.70%
Number (A/M->A/M, MD/D)	268	5.30%
Number (P->P, NC)	493	9.80%
Number (A/M->A/M, NC)	2732	54.30%

Number Increase:

(0<=SLR<1):	296
(SLR>=1):	295
(SLR>=2):	102
(SLR>=3):	29
(SLR>=4):	11

Number Decrease:

(0>=SLR>-1):	258
(SLR<=-1):	640
(SLR<=-2):	227
(SLR<=-3):	48
(SLR<=-4):	4

Spike Controls:

Probe Set	Sig(5')	Det(5')	Sig(M')	Det(M')	Sig(3')	Det(3')	Sig(all)	Sig(3'/5')
AFFX-BIOB	572.2	P	926.9	P	950.2	P	816.44	1.66
AFFX-BIOC	1796.4	P			1413.3	P	1604.87	0.79
AFFX-BIOD	3620.2	P			6565.5	P	5092.84	1.81
AFFX-CRE	13393.2	P			16176.4	P	14784.78	1.21
AFFX-DAP	107.8	P	174.3	P	78.4	P	120.17	0.73
AFFX-LYS	37.6	A	35.2	A	20.6	A	31.11	0.55
AFFX-PHE	247.1	P	24.1	A	14	A	95.07	0.06
AFFX-THR	45	A	56.7	P	101.2	A	67.64	2.25
AFFX-TRP	10	A	21.2	A	5.6	A	12.28	0.55

Report Type:	Comparison Report
Date:	04:43PM 05/04/2007

Filename:	7a&7c.CHP
Probe Array Type:	Bsubtilis
Algorithm:	Statistical
Probe Pair Thr:	8
Controls:	Antisense

Alpha1:	0.04
Alpha2:	0.06
Tau:	0.015
Noise (RawQ):	2.27
Scale Factor (SF):	1.77
TGT Value:	150
Norm Factor (NF):	1
Baseline filename:	Bs7a.CHP
Gamma1H:	0.0025
Gamma1L:	0.0025
Gamma2H:	0.003
Gamma2L:	0.003
Perturbation:	1.1
Baseline Noise (RawQ):	2.51
Baseline Scale Factor (SF):	1.38681579

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	mean	stdev	min	max
Background:	95.95	2.82	87.6	103.2
Noise:	8.32	0.45	6.9	9.5
			count	
Corner+	223		32	
Corner-	17165		32	
Central-	15232		9	

The following data represents probe sets that exceed the probe pair threshold and are not called "No Call".

Total Probe Sets:	5030	
Number Present:	1721	34.20%
Number Absent:	3170	63.00%
Number Marginal:	139	2.80%
Average Signal (P):	973.5	
Average Signal (A):	22.4	
Average Signal (M):	68.7	
Average Signal (All):	349.1	
Number Increase:	538	10.70%
Number Decrease:	924	18.40%
Number MIncrease:	7	0.10%
Number MDecrease:	24	0.50%
Number No Change:	3537	70.30%
Number (A/M->P, MI/I)	117	2.30%
Number (P->A/M, MD/D)	240	4.80%
Number (P->P, MI/I)	398	7.90%
Number (P->P, MD/D)	484	9.60%
Number (A/M->A/M, MI/I)	30	0.60%
Number (A/M->A/M, MD/D)	224	4.50%
Number (P->P, NC)	573	11.40%
Number (A/M->A/M, NC)	2737	54.40%

Number Increase:

(0<=SLR<1):	249
(SLR>=1):	295
(SLR>=2):	103
(SLR>=3):	37
(SLR>=4):	11

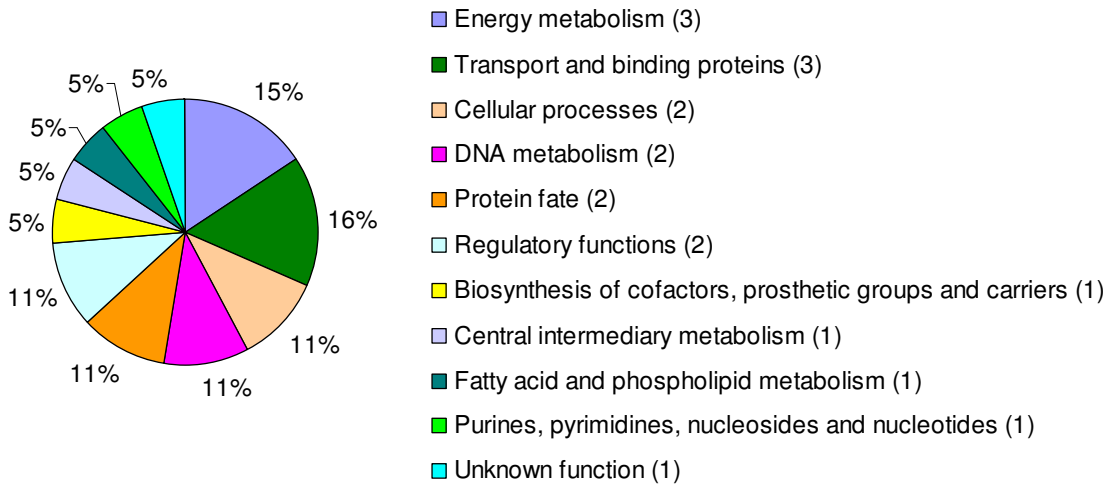
Number Decrease:

(0>=SLR>-1):	382
(SLR<=-1):	559
(SLR<=-2):	186
(SLR<=-3):	35
(SLR<=-4):	2

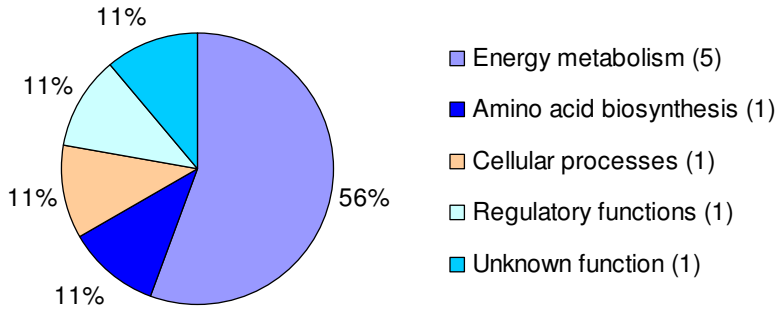
Spike Controls:

Probe Set	Sig(5')	Det(5')	Sig(M')	Det(M')	Sig(3')	Det(3')	Sig(all)	Sig(3'/5')
AFFX-BIOB	794.1	P	1455	P	1422.6	P	1223.89	1.79
AFFX-BIOC	2680.1	P			1842.9	P	2261.47	0.69
AFFX-BIOD	4503.5	P			8424.6	P	6464.05	1.87
AFFX-CRE	17314	P			19968.4	P	18641.17	1.15
AFFX-DAP	95.8	P	143.6	P	81.1	P	106.84	0.85
AFFX-LYS	35.9	A	46.7	A	41	A	41.2	1.14
AFFX-PHE	280.6	P	11	A	16.1	A	102.56	0.06
AFFX-THR	39.5	A	33	A	79.2	A	50.57	2.01
AFFX-TRP	4.8	A	17.4	A	6.2	A	9.45	1.28

Appendix F. Pie charts of differentially regulated genes common to both treatment concentrations.



a)



b)

Figure F1. Functional categories for all differentially regulated genes common to both treatment concentrations. Up-regulated (a) and down-regulated (b).

Primary annotation of *B. subtilis* genome from the JCVI CMR database. Data collected from a Genechip® microarray study described in Section 6.2. Figures in parentheses indicate number of changed genes for treated samples in main functional categories.

Appendix G. Pie charts of differentially regulated genes in only one treatment concentration.

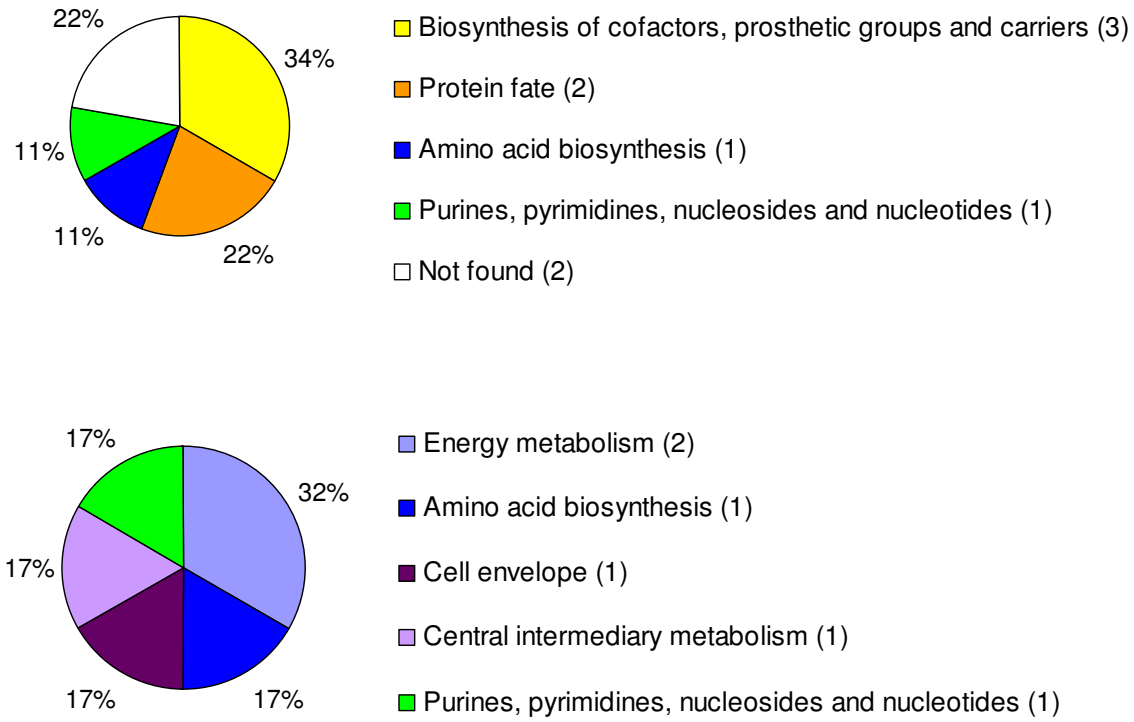
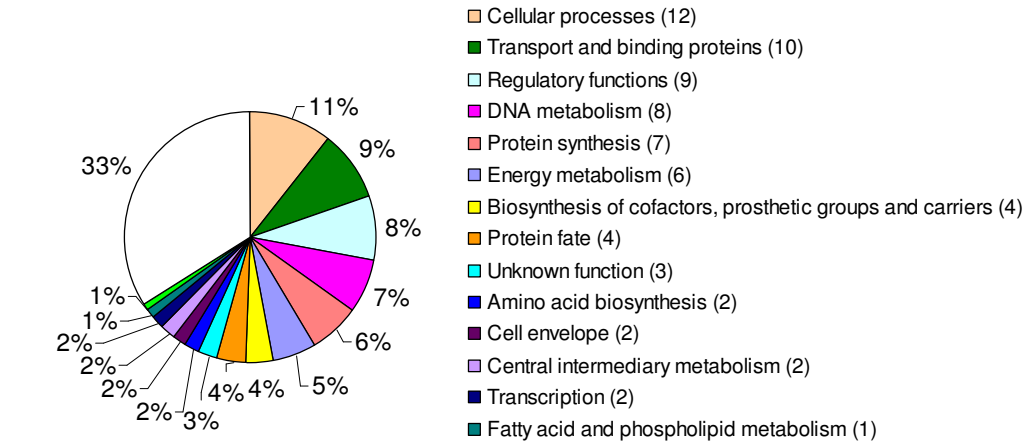
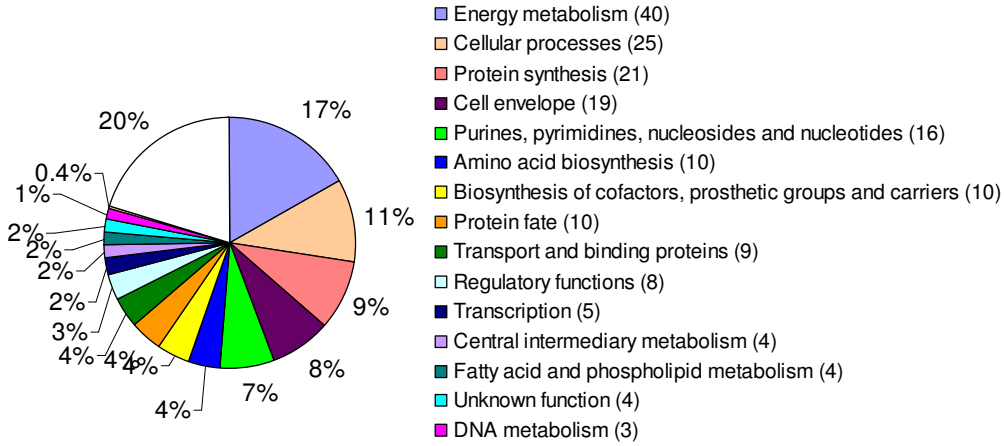


Figure G1. Differentially regulated genes identified in only 2 µg/mL BDM-I treatment. Up-regulated (a) and Down-regulated (b).

Primary annotation of *B. subtilis* genome from the JCVI CMR database. Data collected from a Genechip® microarray study described in Section 6.2. Figures in parentheses indicate number of changed genes for treated samples in main functional categories.



a)



b)

Figure G2. Differentially regulated genes identified in only 16 µg/mL BDM-I treatment. Up-regulated (a) and Down-regulated (b).

Primary annotation of *B. subtilis* genome from the JCVI CMR database. Data collected from a Genechip® microarray study described in Section 6.2. Figures in parentheses indicate number of changed genes for treated samples in main functional categories.

Appendix H: Supplementary Tables - Selected differentially regulated genes and their functional categories as described in JCVI CMR.

Table H1. Genes differentially up-regulated in both treatment concentrations of BDM-I.

Probe set ID	Gene Symbol	Fold change 2 µg/mL	Fold change 16 µg/mL	Main role	Sub role	Common Name
BG12041/ycnE_at	ycnE	28.6	28.2	not found	not found	not found
BG10146/yacH_at	yacH	5.4	10.1	Unknown function	General	similar to hypothetical proteins
BG10148/clpC_at	clpC	4.1	6.9	Protein fate	Degradation of proteins, & glycopeptides	class III stress response-related ATPase
BG10145/ctsR_at	ctsR	4.5	6.2	Regulatory functions	DNA interactions	transcriptional regulator
BG11187/yckK_at	yckK	3.3	6.6	Transport and binding proteins	Amino acids, peptides and amines	similar to glutamine ABC transporter (glutamine-binding protein)
BG10147/yacI_at	yacI	3.6	5.7	Transport and binding proteins; Cellular processes	Carbohydrates, organic alcohols and acids; Toxin production and resistance	similar to creatine kinase
BG11186/yckJ_at	yckJ	2.1	6.7	Transport and binding proteins	Amino acids, peptides and amines	similar to glutamine ABC transporter
BG12583/dnaE_3_at	dnaE	2.7	5.5	DNA metabolism	DNA replication, recombination & repair	dna polymerase iii alpha subunit
BG12583/dnaE_2_at	dnaE	2.2	5.0	DNA metabolism	DNA replication, recombination & repair	dna polymerase iii alpha subunit
BG19016/clpP_at	clpP	4.3	3.3	Protein fate	Degradation of proteins, & glycopeptides	ATP-dependent Clp protease, subunit
BG12398/trxB_at	trxB	2.8	4.3	Energy metabolism	Electron transport	thioredoxin reductase
BG13776/yraA_at	yraA	2.7	3.8	Purines, pyrimidines, nucleosides & nucleotides	Pyrimidine ribonucleotide biosynthesis	similar to hypothetical proteins
BG14144/yvrD_at	yvrD	2.8	3.4	Fatty acid & phospholipid metabolism	Biosynthesis	similar to ketoacyl-carrier protein reductase
BG13023/yhdQ_at	yhdQ	2.5	3.7	Regulatory functions	DNA interactions	similar to transcriptional regulator (MerR)
BG13559/yojG_at	yojG	2.7	3.0	not found	not found	not found
BG12364/yugJ_at	yugJ	3.2	2.4	Energy metabolism; Central intermediary metabolism	Electron transport; Fermentation; Glycolysis/gluconeogenesis; TCA cycle	similar to NADH-dependent butanol dehydrogenase
BG13434/ymzA_at	ymzA	2.1	3.2	not found	not found	not found
BG13022/yhdP_at	yhdP	2.0	2.9	Cellular processes	Other	similar to hemolysin
BG10348/trxA_at	trxA	2.1	2.5	Energy metabolism	Electron transport	thioredoxin
BG11671/yqfY_at	yqfY	2.0	2.0	Biosynthesis of cofactors, prosthetic groups,	Other	similar to peptidoglycan acetylation

Table H2. Genes differentially down-regulated in both treatment concentrations of BDM-I.

Probe set ID	Gene Symbol	Fold change 2 µg/mL	Fold change 16 µg/mL	Main role	Sub role	Common Name
BG10112/spoVG_at	spoVG	6.9	17.4	Cellular processes	Other	required for spore cortex synthesis (stage V)
BG13246/yknZ_at	yknZ	2.2	6.3	not found	not found	not found
BG10272/odhA_1_at	odhA	2.1	6.2	Energy metabolism	TCA cycle	2-oxoglutarate dehydrogenase (E1 subunit)
BG11156/yxjG_at	yxjG	2.2	5.8	not found	not found	not found
BG12681/sucD_at	sucD	3.2	3.7	Energy metabolism	TCA cycle	succinyl-CoA synthetase (alpha subunit)
BG13416/ymaH_at	ymaH	2.5	4.7	Regulatory functions	Other	similar to host factor-1 protein
BG11570/ybbI_at	ybbI	2.1	3.8	Unknown function	General	similar to hypothetical proteins
BG10673/ilvA_at	ilvA	2.2	3.5	Amino acid biosynthesis	Other	threonine dehydratase
BG10722/rocD_at	rocD	2.1	2.9	Energy metabolism	Amino acids & amines	acetylornithine aminotransferase
BG10353/sdhB_at	sdhB	2.3	2.7	Energy metabolism	TCA cycle	succinate dehydrogenase (iron-sulfur protein)
BG10478/citB_1_at	citB	2.2	2.5	Energy metabolism	TCA cycle	aconitate hydratase

not found, Not found in the JCVI Primary annotation database

Table H3. Genes differentially up-regulated by 2 µg/mL BDM-I only.

Probe set ID	Gene Symbol	Fold change 2 µg/mL	Fold change 16 µg/mL	Main role	Sub role
BG10520/ribA_at	ribA	2.5	Amino acid biosynthesis	Histidine family	GTP cyclohydrolase II and 3,4-dihydroxy-2-butanone 4-phosphate synthase
BG10520/ribA_at	ribA	2.5	Biosynthesis of cofactors, prosthetic groups and carriers	Folic acid; Riboflavin, FMN and FAD; Molybdopterin	GTP cyclohydrolase II and 3,4-dihydroxy-2-butanone 4-phosphate synthase
BG10519/ribB_at	ribB	3.4	Biosynthesis of cofactors, prosthetic groups and carriers	Riboflavin, FMN and FAD	riboflavin synthase (alpha subunit)
BG10521/ribH_at	ribH	2.3	Biosynthesis of cofactors, prosthetic groups and carriers	Riboflavin, FMN and FAD	riboflavin synthase (beta subunit)
BG10522/ribT_at	ribT	2.1	not found	not found	not found
BG10517/ypuE_i_at	ypuE	2.3	not found	not found	not found
BG10664/dnaK_at	dnaK	2.2	Protein fate	Protein folding and stabilization	class I heat-shock protein (chaperonin)
BG10663/grpE_at	grpE	2.2	Protein fate	Protein folding and stabilization	heat-shock protein
BG11426/ymaA_at	ymaA	2.0	Purines, pyrimidines, nucleosides and nucleotides	2'-Deoxyribonucleotide metabolism	similar to ribonucleoprotein

not found, Not found in the JCVI Primary annotation database

Table H4. Genes differentially down-regulated by 2 µg/mL BDM-I only.

Probe set ID	Gene Symbol	Fold change 2 µg/mL	Fold change 16 µg/mL	Main role	Sub role
BG10460/hom_at	hom	2.1	Amino acid biosynthesis	Other	homoserine dehydrogenase
BG10797/wapA_3_at	wapA	2.1	Cell envelope	Other	cell wall-associated protein precursor (CWBP200, 105, 62)
BG13379/ylnC_at	ylnC	2.8	Central intermediary metabolism	Sulfur metabolism	similar to adenylylsulfate kinase
BG10822/atpC_at	atpC	2.1	Energy metabolism	ATP-proton motive force interconversion	ATP synthase (subunit epsilon)
BG12001/lctP_at	lctP	2.2	Energy metabolism	Glycolysis/gluconeogenesis	L-lactate permease
BG10718/pyrD_at	pyrD	2.4	Purines, pyrimidines, nucleosides and nucleotides	Pyrimidine ribonucleotide biosynthesis	dihydroorotate dehydrogenase

not found, Not found in the JCVI Primary annotation database

Table H5. Genes differentially up-regulated by 16 µg/mL BDM-I only.

Probe set ID	Gene Symbol	Fold change 2 µg/mL	Fold change 16 µg/mL	Main role	Sub role
BG10864/mrgA_at	mrgA	18.5	not found	not found	not found
BG12034/yclN_at	yclN	12.3	Transport and binding proteins	Other	similar to ferrichrome ABC transporter (permease)
BG12863/yetG_at	yetG	9.6	not found	not found	not found
BG14055/yuzF_at	yuzF	9.2	not found	not found	not found
BG12291/yrhB_at	yrhB	7.9	Amino acid biosynthesis	Other	similar to cystathionine gamma-synthase
BG14107/yvgY_at	yvgY	7.8	Transport and binding proteins	Other	similar to mercuric transport protein
BG14108/yvgZ_at	yvgZ	6.5	not found	not found	not found
BG11828/ynfC_at	ynfC	6.4	not found	not found	not found
BG14098/yvgP_at	yvgP	6.3	Transport and binding proteins	Cations and iron carrying compounds	similar to hypothetical proteins
BG12325/yshA_at	yshA	5.5	not found	not found	not found
BG12878/yfhC_at	yfhC	5.1	not found	not found	not found
BG10133/yacB_at	yacB	5.1	not found	not found	not found
BG10680/mecA_at	mecA	5.0	Cellular processes	DNA transformation	negative regulator of competence
BG12612/lmrA_at	lmrA	5.0	Regulatory functions	DNA interactions	transcriptional regulator
BG13800/yrrU_at	yrrU	4.9	Purines, pyrimidines, nucleosides and nucleotides; Unknown function	Salvage of nucleosides and nucleotides, Other, General	similar to purine nucleoside phosphorylase
BG13816/yrzF_at	yrzF	4.9	not found	not found	not found
BG10829/ylxM_at	ylxM	4.7	not found	not found	not found
BG12495/ywoH_at	ywoH	4.6	Regulatory functions	DNA interactions	similar to transcriptional regulator (MarR family)
BG11067/ybxA_at	ybxA	4.1	Transport and binding proteins	Other	alternate gene name: ybaD similar to ABC
BG13138/yjbI_at	yjbI	4.1	not found	not found	not found
BG12223/ygaC_at	ygaC	4.1	not found	not found	not found
BG12370/yugP_at	yugP	4.0	not found	not found	not found
BG13832/ytcF_at	ytcF	3.9	Central intermediary metabolism	Polyamine biosynthesis	unknown
BG10830/ffh_at	ffh	3.7	Protein fate	Protein and peptide secretion and trafficking	signal recognition particle
BG13314/ykvL_at	ykvL	3.7	Biosynthesis of cofactors, prosthetic groups and carriers	Other	similar to coenzyme PQQ synthesis
BG13379/ylnC_at	ylnC	3.6	Central intermediary metabolism	Sulfur metabolism	similar to adenylylsulfate kinase

BG13312/ykvJ_at	ykvJ	3.6	Unknown function	General	similar to hypothetical proteins
BG11971/rplT_at	rplT	3.6	Protein synthesis	Ribosomal proteins: synthesis and modification	ribosomal protein L20
BG11638/yqeJ_at	yqeJ	3.6	Biosynthesis of cofactors, prosthetic groups and carriers	Pyridine nucleotides	similar to hypothetical proteins
BG10060/thdF_at	thdF	3.5	Protein synthesis	tRNA and rRNA base modification	thiophen and furan oxidation
BG10662/hrcA_at	hrcA	3.5	Regulatory functions	DNA interactions	transcriptional regulator
BG13787/yrrA_at	yrrA	3.4	Protein synthesis	tRNA and rRNA base modification	similar to hypothetical proteins
BG12535/ywtD_at	ywtD	3.4	Cell envelope	Biosynthesis and degradation of murein sacculus and peptidoglycan	similar to murein hydrolase
BG14160/yvzD_at	yvzD	3.4	not found	not found	not found
BG14111/yvjD_at	yvjD	3.3	not found	not found	not found
BG10134/yacC_at	yacC	3.3	Protein fate	Protein folding and stabilization	similar to hypothetical proteins
BG12399/yvcI_at	yvcI	3.3	DNA metabolism	DNA replication, recombination and repair	similar to mutator MutT protein
BG13137/yjbH_at	yjbH	3.2	not found	not found	not found
BG13767/ypzE_at	ypzE	3.2	not found	not found	not found
BG10399/flgM_at	flgM	3.2	Cellular processes	Chemotaxis and motility	anti-sigma factor repressor of sigma-D-dependent transcription
BG10399/flgM_at	flgM	3.2	Regulatory functions	DNA interactions	anti-sigma factor repressor of sigma-D-dependent transcription
BG10501/csbA_at	csbA	3.2	Cellular processes	Other	putative membrane protein
BG11637/yqeI_at	yqeI	3.1	not found	not found	not found
BG11989/ybaE_at	ybaE	3.1	Transport and binding proteins	Other	similar to ABC transporter (ATP-binding protein)
BG13866/ytjB_at	ytjB	3.1	Cellular processes	Other	unknown
BG13783/yrbC_at	yrbC	3.1	not found	not found	not found
BG11992/truA_at	truA	3.0	Protein synthesis	tRNA and rRNA base modification	pseudouridylate synthase I
BG10786/sspA_at	sspA	3.0	Cellular processes	Adaptations to atypical conditions	small acid-soluble spore protein (alpha-type SASP)
BG11573/sigW_at	sigW	3.0	DNA metabolism	DNA replication, recombination and repair	RNA polymerase ECF-type sigma factor (sigma-W)
BG11573/sigW_at	sigW	3.0	Transcription	DNA-dependent RNA polymerase, Other	RNA polymerase ECF-type sigma factor (sigma-W)
BG10861/mcpA_at	mcpA	2.9	Cellular processes	Chemotaxis and motility	methyl-accepting chemotaxis protein
BG11202/ykyB_at	ykyB	2.9	not found	not found	not found
BG10103/yabE_at	yabE	2.9	not found	not found	not found
BG12083/ydbP_at	ydbP	2.9	Energy metabolism	ATP-proton motive force interconversion	similar to thioredoxin
BG11574/ybbM_at	ybbM	2.8	not found	not found	not found
BG10150/yacK_at	yacK	2.8	not found	not found	not found
BG12656/polA_at	polA	2.8	DNA metabolism	DNA replication, recombination and repair	DNA polymerase I
BG10589/ywcG_at	ywcG	2.8	not found	not found	not found

BG11220/rplJ_at	rplJ	2.8	Protein synthesis	Ribosomal proteins: synthesis and modification	ribosomal protein L10 (BL5)
BG12632/nifZ_at	nifZ	2.8	Cellular processes	Other	NifS protein homolog
BG12957/yfmF_at	yfmF	2.7	Transport and binding proteins	Other	similar to ferrichrome ABC transporter (ATP-binding protein)
BG13467/ynzD_at	ynzD	2.6	not found	not found	not found
BG11635/yqeG_at	yqeG	2.6	not found	not found	not found
BG11012/pssA_at	pssA	2.6	Fatty acid and phospholipid metabolism	Biosynthesis	phosphatidylserine synthase
BG10130/yacA_at	yacA	2.6	Cellular processes	Cell division	similar to cell-cycle protein
BG10151/yacL_at	yacL	2.6	not found	not found	not found
BG11562/ybaR_at	ybaR	2.6	Protein fate	Degradation of proteins, peptides and glycopeptides	similar to hypothetical proteins
BG11562/ybaR_at	ybaR	2.6	Transport and binding proteins	Cations and iron carrying compounds	similar to hypothetical proteins
BG11337/phoH_at	phoH	2.6	not found	not found	not found
BG11766/yqkL_at	yqkL	2.6	Regulatory functions	Other	similar to transcriptional regulator (Fur family)
BG11522/aroD_at	aroD	2.6	Amino acid biosynthesis	Aromatic amino acid family	shikimate 5-dehydrogenase
BG10942/ywlF_at	ywlF	2.5	Energy metabolism	Pentose phosphate pathway	alternate gene name: ipc-32d similar to ribose
BG10654/csrA_at	csrA	2.5	Energy metabolism	Glycolysis/gluconeogenesis	carbon storage regulator
BG10654/csrA_at	csrA	2.5	Regulatory functions	RNA interactions; Other	carbon storage regulator
BG11434/ypbH_at	ypbH	2.5	Cellular processes	DNA transformation	similar to negative regulation of competence MecA
BG10699/degR_at	degR	2.5	Regulatory functions	Other	activation of degradative enzymes (aprE, nprE, sacB)
BG12842/yerP_2_at	yerP	2.5	Cellular processes	Detoxification	similar to acriflavin resistance protein
BG10097/yabA_at	yabA	2.5	not found	not found	not found
BG12205/ydiH_at	ydiH	2.5	DNA metabolism	DNA replication, recombination and repair	unknown
BG10149/sms_at	sms	2.5	DNA metabolism	DNA replication, recombination and repair	DNA repair protein homolog
BG11447/yppF_at	yppF	2.5	not found	not found	not found
BG12332/ysmB_at	ysmB	2.4	Regulatory functions	DNA interactions	similar to transcriptional regulator (MarR family)
BG14045/yutI_at	yutI	2.4	Cellular processes	Other	similar to NifU protein homolog
BG12451/yviF_at	yviF	2.4	Cell envelope	Other	similar to hypothetical proteins from B. subtilis
BG10678/lexA_at	lexA	2.4	DNA metabolism	DNA replication, recombination and repair	transcriptional regulator
BG10678/lexA_at	lexA	2.4	Regulatory functions	DNA interactions	transcriptional regulator
BG12745/ybfQ_at	ybfQ	2.3	not found	not found	not found
BG13377/ylnA_at	ylnA	2.3	Transport and binding proteins	Amino acids, peptides and amines	similar to anion permease
BG12666/rnh_at	rnh	2.3	Transcription	Degradation of RNA	ribonuclease H
BG10059/gidA_at	gidA	2.3	Unknown function	General	glucose-inhibited division protein

BG12656/polA_1_at	polA	2.3	DNA metabolism	DNA replication, recombination and repair	DNA polymerase I
BG12204/ydiG_at	ydiG	2.3	Biosynthesis of cofactors, prosthetic groups and carriers	Molybdopterin	similar to molybdopterin precursor biosynthesis
BG10096/yaaT_at	yaaT	2.3	Protein fate	Degradation of proteins, peptides and glycopeptides	similar to signal peptidase II
BG10688/motA_at	motA	2.2	Cellular processes	Chemotaxis and motility	motility protein A
BG10104/yabF_at	yabF	2.2	Unknown function	General	similar to hypothetical proteins
BG12403/crh_at	crh	2.2	Energy metabolism	Other	catabolite repression HPr-like protein
BG12227/ygaG_at	ygaG	2.2	Energy metabolism	Sugars	similar to transcriptional regulator (Fur family)
BG10334/ysxB_at	ysxB	2.2	not found	not found	not found
BG11022/ypmR_at	ypmR	2.2	not found	not found	not found
BG11970/rplM_at	rplM	2.2	Protein synthesis	Ribosomal proteins: synthesis and modification	ribosomal protein L13
BG10372/rpsD_at	rpsD	2.2	Protein synthesis	Ribosomal proteins: synthesis and modification	ribosomal protein S4 (BS4)
BG11645/yqeU_at	yqeU	2.1	not found	not found	not found
BG12969/yfmR_at	yfmR	2.1	Transport and binding proteins	Other	similar to ABC transporter (ATP-binding protein)
BG13929/ytvB_at	ytvB	2.1	not found	not found	not found
BG11371/opuAB_at	opuAB	2.1	Transport and binding proteins	Amino acids, peptides and amines; Cations and iron carrying compounds; Unknown substrate	glycine betaine ABC transporter (permease)
BG11716/yqiG_at	yqiG	2.1	Energy metabolism	Aerobic; Electron transport	similar to NADH-dependent flavin oxidoreductase
BG13827/ytbJ_at	ytbJ	2.1	Biosynthesis of cofactors, prosthetic groups and carriers	Thiamine	similar to hypothetical proteins
BG10951/ygxA_at	ygxA	2.1	not found	not found	not found
BG12656/polA_2_at	polA	2.1	DNA metabolism	DNA replication, recombination and repair	DNA polymerase I
BG11641/yqeM_at	yqeM	2.0	not found	not found	not found
BG12920/yfkC_at	yfkC	2.0	not found	not found	not found

not found, Not found in the JCVI Primary annotation database

Table H6. Genes differentially down-regulated by 16 µg/mL BDM-I only.

Probe set ID	Gene Symbol	Fold change 2 µg/mL	Fold change 16 µg/mL	Main role	Sub role
BG10240/fliF_at	fliF	13.2	Cellular processes	Chemotaxis and motility	flagellar basal-body M-ring protein
BG19003/lctE_at	lctE	13.0	Energy metabolism	Aerobic, Glycolysis/gluconeogenesis	L-lactate dehydrogenase
BG10706/purQ_at	purQ	11.7	Purines, pyrimidines, nucleosides and nucleotides	Purine ribonucleotide biosynthesis	phosphoribosylformylglycinamidine synthetase I
BG10704/yexA_at	yexA	10.7	not found	not found	not found
BG11062/pgk_at	pgk	10.6	Energy metabolism	Glycolysis/gluconeogenesis	phosphoglycerate kinase
BG14170/yxzG_at	yxzG	10.2	not found	not found	not found
BG12605/hisS_at	hisS	10.0	Protein synthesis	tRNA aminoacylation	histidyl-tRNA synthetase
BG12838/yerL_at	yerL	9.7	Protein synthesis	tRNA aminoacylation	alternate gene name: yedA similar to hypothetical
BG12660/pycA_3_at	pycA	8.4	Energy metabolism	Glycolysis/gluconeogenesis	pyruvate carboxylase
BG10740/yvyD_at	yvyD	7.9	Protein synthesis	Translation factors	alternate gene name: yvil similar to ribosomal
BG11846/wprA_at	wprA	7.9	Cell envelope	Other	cell wall-associated protein precursor (CWBP23, CWBP52)
BG10276/hbs_at	hbs	7.8	DNA metabolism	DNA replication, recombination and repair; Chromosome-associated proteins	non-specific DNA-binding protein HBsu
BG10707/purF_at	purF	7.5	Purines, pyrimidines, nucleosides and nucleotides	Purine ribonucleotide biosynthesis	phosphoribosylpyrophosphate amidotransferase
BG13785/yrbF_at	yrbF	7.3	Protein fate	Protein and peptide secretion and trafficking	similar to hypothetical proteins
BG11384/accC_at	accC	7.1	Fatty acid and phospholipid metabolism	Biosynthesis	acetyl-CoA carboxylase subunit (biotin carboxylase subunit)
BG10897/tpi_at	tpi	7.1	Energy metabolism	Glycolysis/gluconeogenesis	triose phosphate isomerase
BG11023/yvcE_at	yvcE	6.9	Cell envelope	Biosynthesis and degradation of murein sacculus and peptidoglycan	alternate gene name: yzkA similar to cell
BG11846/wprA_1_at	wprA	6.9	Cell envelope	Other	cell wall-associated protein precursor (CWBP23, CWBP52)
BG10703/purC_at	purC	6.6	Purines, pyrimidines, nucleosides and nucleotides	Purine ribonucleotide biosynthesis	phosphoribosylaminoimidazole succinocarboxamide synthetase
BG12572/aspS_at	aspS	6.6	Protein synthesis	tRNA aminoacylation	aspartyl-tRNA synthetase
BG13245/yknY_at	yknY	6.6	Transport and binding proteins	Other	similar to ABC transporter (ATP-binding protein)
BG11247/tkt_at	tkt	6.5	Energy metabolism	Pentose phosphate pathway	transketolase

BG11135/yxiF_at	yxiF	6.3	not found	not found	not found
BG10705/purL_at	purL	6.2	Purines, pyrimidines, nucleosides and nucleotides	Other	phosphoribosylformylglycinamidine synthetase II
BG11816/yktA_at	yktA	6.2	not found	not found	not found
BG11460/efp_at	efp	6.1	Protein synthesis	Translation factors	elongation factor P
BG13236/ykgB_at	ykgB	6.1	not found	not found	not found
BG10815/atpB_at	atpB	6.0	Energy metabolism	ATP-proton motive force interconversion	ATP synthase (subunit a)
BG13395/yloU_at	yloU	6.0	Cellular processes	Adaptations to atypical conditions	similar to alkaline-shock protein
BG10816/atpE_at	atpE	6.0	Energy metabolism	ATP-proton motive force interconversion	ATP synthase (subunit c)
BG10622/rocA_at	rocA	5.8	Energy metabolism	Amino acids and amines	pyrroline-5 carboxylate dehydrogenase
BG11138/yxiI_at	yxiI	5.6	not found	not found	not found
BG10817/atpF_at	atpF	5.5	Energy metabolism	ATP-proton motive force interconversion	ATP synthase (subunit b)
BG13392/yloQ_at	yloQ	5.5	not found	not found	not found
BG12839/yerM_at	yerM	5.5	Protein synthesis	tRNA aminoacylation	alternate gene name: yedB similar to amidase
BG11709/yqhY_at	yqhY	5.5	not found	not found	not found
BG13152/yjbW_at	yjbW	5.3	Fatty acid and phospholipid metabolism	Biosynthesis	similar to enoyl- acyl-carrier protein reductase
BG10241/fliG_at	fliG	5.3	Cellular processes	Chemotaxis and motility	flagellar motor switch protein
BG12366/pgi_at	pgi	5.3	Energy metabolism	Glycolysis/gluconeogenesis	glucose-6-phosphate isomerase
BG13934/ytwP_at	ytwP	5.1	Energy metabolism	Sugars	unknown
BG11708/yqhT_at	yqhT	5.0	Protein fate	Degradation of proteins, peptides and glycopeptides	similar to Xaa-Pro dipeptidase
BG11080/pbuX_at	pbuX	4.8	not found	not found	not found
BG14161/ywiB_at	ywiB	4.8	not found	not found	not found
BG10231/ftsA_at	ftsA	4.8	Cellular processes	Cell division	cell-division protein
BG10402/gtaB_at	gtaB	4.6	Cell envelope	Biosynthesis and degradation of surface polysaccharides and lipopolysaccharides	UTP-glucose-1-phosphate uridylyltransferase
BG11707/yqhS_at	yqhS	4.6	Amino acid biosynthesis	Aromatic amino acid family	similar to 3-dehydroquinate dehydratase
BG10702/purB_at	purB	4.6	Purines, pyrimidines, nucleosides and nucleotides	Purine ribonucleotide biosynthesis	adenylosuccinate lyase
BG11738/yqjI_at	yqjI	4.5	Energy metabolism	Pentose phosphate pathway	similar to 6-phosphogluconate dehydrogenase (pentose phosphate)
BG10711/purD_at	purD	4.5	Purines, pyrimidines, nucleosides and nucleotides	Purine ribonucleotide biosynthesis	phosphoribosylglycinamide synthetase
BG11005/ypfD_at	ypfD	4.5	Protein synthesis	Ribosomal proteins: synthesis and modification	alternate gene name: jofD similar to ribosomal

BG11502/ypmB_at	ypmB	4.4	not found	not found	not found
BG13403/ylqD_at	ylqD	4.3	not found	not found	not found
BG10989/kinC_at	kinC	4.3	Regulatory functions	Protein interactions	two-component sensor histidine kinase
BG12584/dps_at	dps	4.3	Cellular processes	Adaptations to atypical conditions	alternate gene name: ytkB stress- and starvation-induced
BG10898/pgm_at	pgm	4.2	Energy metabolism	Glycolysis/gluconeogenesis	phosphoglycerate mutase
BG10797/wapA_2_at	wapA	4.2	Cell envelope	Other	cell wall-associated protein precursor (CWBP200, 105, 62)
BG10281/gerCC_at	gerCC	4.1	Biosynthesis of cofactors, prosthetic groups and carriers	Other	heptaprenyl diphosphate synthase component II
BG10856/citC_at	citC	4.1	Energy metabolism	TCA cycle	isocitrate dehydrogenase
BG10818/atpH_at	atpH	4.1	Energy metabolism	ATP-proton motive force interconversion	ATP synthase (subunit delta)
BG10282/ndk_at	ndk	4.0	Purines, pyrimidines, nucleosides and nucleotides	Other	nucleoside diphosphate kinase
BG10230/sbp_at	sbp	3.9	not found	not found	not found
BG10437/bglC_at	bglC	3.9	Energy metabolism	Other	endo-1,4-beta-glucanase
BG11141/yxiL_at	yxiL	3.9	not found	not found	not found
BG13801/yrvB_at	yrvB	3.9	not found	not found	not found
BG11137/yxiH_at	yxiH	3.9	not found	not found	not found
BG13215/yjoA_at	yjoA	3.9	not found	not found	not found
BG10948/glmS_at	glmS	3.9	Central intermediary metabolism	Amino sugars	L-glutamine-D-fructose-6-phosphate amidotransferase
BG10797/wapA_1_at	wapA	3.9	Cell envelope	Other	cell wall-associated protein precursor (CWBP200, 105, 62)
BG10239/fliE_at	fliE	3.8	Cellular processes	Chemotaxis and motility	flagellar hook-basal body protein
BG11739/yqjJ_at	yqjJ	3.8	Energy metabolism	Pentose phosphate pathway	similar to glucose-6-phosphate 1-dehydrogenase (pentose phosphate)
BG10874/pheS_at	pheS	3.8	Protein synthesis	tRNA aminoacylation	phenylalanyl-tRNA synthetase (alpha subunit)
BG10255/cheB_at	cheB	3.8	Regulatory functions	DNA interactions	methyl-accepting chemotaxis proteins (MCP)-glutamate methylesterase and two-component
BG10990/ykqA_at	ykqA	3.7	not found	not found	not found
BG14150/yvrM_at	yvrM	3.7	not found	not found	not found
BG13054/yhfI_at	yhfI	3.6	Unknown function	Enzymes of unknown specificity	similar to hypothetical proteins
BG10797/wapA_4_at	wapA	3.6	Cell envelope	Other	cell wall-associated protein precursor (CWBP200, 105, 62)
BG10468/ald_at	ald	3.6	Energy metabolism	Amino acids and amines	L-alanine dehydrogenase
BG10469/yuxI_at	yuxI	3.5	Transcription	Transcription factors	alternate gene name: yukI
BG11386/citH_at	citH	3.5	Transport and binding proteins	Other	malate dehydrogenase

BG10797/wapA_at	wapA	3.5	Cell envelope	Other	cell wall-associated protein precursor (CWBP200, 105, 62)
BG10402/gtaB_g_at	gtaB	3.5	Cell envelope	Biosynthesis and degradation of surface polysaccharides and lipopolysaccharides	UTP-glucose-1-phosphate uridylyltransferase
BG10819/atpA_at	atpA	3.4	Energy metabolism	ATP-proton motive force interconversion	ATP synthase (subunit alpha)
BG13970/yuiE_at	yuiE	3.4	Protein fate	Degradation of proteins, peptides and glycopeptides	similar to leucyl aminopeptidase
BG14152/yvrP_at	yvrP	3.4	Transport and binding proteins	Other	similar to hypothetical proteins from B. subtilis
BG13244/yknX_at	yknX	3.4	not found	not found	not found
BG19025/tsf_at	tsf	3.4	Protein synthesis	Translation factors	elongation factor Ts
BG10325/ysxA_at	ysxA	3.4	Cell envelope	Surface structures; Other	similar to DNA repair protein
BG12460/ywhF_at	ywhF	3.4	Central intermediary metabolism	Polyamine biosynthesis	similar to spermidine synthase
BG10509/serA_at	serA	3.3	Amino acid biosynthesis	Serine family	phosphoglycerate dehydrogenase
BG10686/menB_at	menB	3.3	Biosynthesis of cofactors, prosthetic groups and carriers	Menaquinone and ubiquinone	dihydroxynapthoic acid synthetase
BG11733/yqjD_at	yqjD	3.3	Fatty acid and phospholipid metabolism	Degradation; Other	similar to propionyl-CoA carboxylase
BG11657/glyQ_at	glyQ	3.3	Protein synthesis	tRNA aminoacylation	glycyl-tRNA synthetase (alpha subunit)
BG13349/ylaL_at	ylaL	3.3	Protein synthesis	tRNA aminoacylation	unknown
BG11856/ylmH_at	ylmH	3.3	Cellular processes	Cell division	similar to cell-division protein
BG11474/yydA_at	yydA	3.3	not found	not found	not found
BG10422/groES_at	groES	3.3	Protein fate	Protein folding and stabilization	class I heat-shock protein (molecular chaperonin)
BG10899/eno_at	eno	3.2	Energy metabolism	Glycolysis/gluconeogenesis	enolase
BG13814/yrzD_at	yrzD	3.2	not found	not found	not found
BG10920/yvyC_at	yvyC	3.2	Cellular processes	Chemotaxis and motility	alternate gene name: yviH similar to flagellar
BG12946/yflK_at	yflK	3.2	not found	not found	not found
BG11536/acpA_at	acpA	3.2	Fatty acid and phospholipid metabolism	Biosynthesis	acyl carrier protein
BG13432/ymfL_at	ymfL	3.2	Unknown function	General	unknown
BG11331/drm_at	drm	3.2	Purines, pyrimidines, nucleosides and nucleotides	Salvage of nucleosides and nucleotides	phosphodeoxyribomutase
BG13966/yueK_at	yueK	3.2	Biosynthesis of cofactors, prosthetic groups and carriers	Pyridine nucleotides	similar to nicotinate phosphoribosyltransferase
BG11034/yfjL_at	yfjL	3.1	Transcription	Transcription factors	alternate gene name: yztA
BG13390/yloO_at	yloO	3.1	Protein fate	Protein modification and repair	similar to hypothetical proteins

BG11211/ypjF_at	ypjF	3.1	Energy metabolism	Other	alternate gene name: jojF similar to hypothetical
BG12079/ydbL_at	ydbL	3.1	not found	not found	not found
BG12378/yukE_at	yukE	3.1	not found	not found	not found
BG14127/yvoE_at	yvoE	3.1	Energy metabolism	Sugars	similar to phosphoglycolate phosphatase
BG12060/ydaM_at	ydaM	3.0	Energy metabolism	Other	similar to cellulose synthase
BG10701/purK_at	purK	3.0	Purines, pyrimidines, nucleosides and nucleotides	Purine ribonucleotide biosynthesis	phosphoribosylaminoimidazole carboxylase II
BG12201/ydiD_at	ydiD	3.0	Protein synthesis	Ribosomal proteins: synthesis and modification	similar to ribosomal-protein-alanine N-acetyltransferase
BG10708/purM_at	purM	2.9	Purines, pyrimidines, nucleosides and nucleotides	Purine ribonucleotide biosynthesis	phosphoribosylaminoimidazole synthetase
BG10425/glnA_at	glnA	2.9	Amino acid biosynthesis	Glutamate family	glutamine synthetase
BG10797/wapA_5_at	wapA	2.9	Cell envelope	Other	cell wall-associated protein precursor (CWBP200, 105, 62)
BG13391/yloP_at	yloP	2.9	Cell envelope	Surface structures; Other	similar to protein kinase
BG12623/moeA_at	moeA	2.9	Biosynthesis of cofactors, prosthetic groups and carriers	Other	molybdopterin biosynthesis protein
BG10352/sdhA_at	sdhA	2.9	Energy metabolism	TCA cycle	succinate dehydrogenase (flavoprotein subunit)
BG10351/sdhC_at	sdhC	2.8	Energy metabolism	Electron transport; TCA cycle	succinate dehydrogenase (cytochrome b558 subunit)
BG10054/spo0J_at	spo0J	2.8	Cellular processes	Adaptations to atypical conditions	antagonist of Soj
BG12800/ydjI_at	ydjI	2.8	not found	not found	not found
BG10237/flgB_at	flgB	2.8	Cellular processes	Chemotaxis and motility	flagellar basal-body rod protein
BG13160/yjcG_at	yjcG	2.8	not found	not found	not found
BG11494/ybaL_at	ybaL	2.8	not found	not found	not found
BG10945/upp_at	upp	2.8	Purines, pyrimidines, nucleosides and nucleotides	Salvage of nucleosides and nucleotides	uracil phosphoribosyltransferase
BG14143/yvrC_at	yvrC	2.8	Transport and binding proteins	Other	similar to iron-binding protein
BG11792/ileS_1_at	ileS	2.8	Protein synthesis	tRNA aminoacylation	isoleucyl-tRNA synthetase
BG13203/yjlD_at	yjlD	2.8	Energy metabolism	ATP-proton motive force interconversion	similar to NADH dehydrogenase
BG10814/atpI_at	atpI	2.8	not found	not found	not found
BG14128/yvoF_at	yvoF	2.7	Energy metabolism	Other	similar to O-acetyltransferase
BG10412/fbaA_at	fbaA	2.7	Energy metabolism	Glycolysis/gluconeogenesis	fructose-1,6-bisphosphate aldolase
BG10243/fliI_at	fliI	2.7	Cellular processes	Chemotaxis and motility	flagellar-specific ATP synthase
BG13361/ylbI_at	ylbI	2.7	Biosynthesis of cofactors, prosthetic groups and carriers	Pantothenate and coenzyme A	similar to lipopolysaccharide core biosynthesis

BG10238/flgC_at	flgC	2.7	Cellular processes	Chemotaxis and motility	flagellar basal-body rod protein
BG10963/proB_at	proB	2.7	Amino acid biosynthesis	Glutamate family	gamma-glutamyl kinase
BG11341/argS_at	argS	2.7	Protein synthesis	tRNA aminoacylation	arginyl-tRNA synthetase
BG10219/ylxA_at	ylxA	2.7	Unknown function	Enzymes of unknown specificity	alternate gene name: yllC similar to hypothetical
BG10413/ywjH_at	ywjH	2.7	Energy metabolism	Pentose phosphate pathway	similar to transaldolase (pentose phosphate)
BG10394/yviA_at	yviA	2.7	not found	not found	not found
BG10224/mraY_at	mraY	2.7	Cell envelope	Biosynthesis and degradation of murein sacculus and peptidoglycan	phospho-N-acetylmuramoyl-pentapeptide transferase
BG11491/pnpA_at	pnpA	2.7	Purines, pyrimidines, nucleosides and nucleotides	Other	polynucleotide phosphorylase (PNPase)
BG14146/yvrG_at	yvrG	2.6	Regulatory functions	Protein interactions	similar to two-component sensor histidine kinase [YvrH]
BG12640/opuCD_at	opuCD	2.6	Transport and binding proteins	Amino acids, peptides and amines	glycine betaine/carnitine/choline ABC transporter (membrane protein)
BG10837/feuC_at	feuC	2.6	Cell envelope	Other	integral membrane protein
BG10837/feuC_at	feuC	2.6	Transport and binding proteins	Cations and iron carrying compounds	integral membrane protein
BG11539/ftsY_at	ftsY	2.6	Protein fate	Protein and peptide secretion and trafficking	signal recognition particle (docking protein)
BG11527/bioF_at	bioF	2.6	Biosynthesis of cofactors, prosthetic groups and carriers	Biotin	8-amino-7-oxononanoate synthase
BG11425/yllB_at	yllB	2.6	not found	not found	not found
BG10294/aroE_at	aroE	2.6	Amino acid biosynthesis	Aromatic amino acid family	5-enolpyruvoylshikimate-3-phosphate synthase
BG11207/dapB_at	dapB	2.6	Amino acid biosynthesis	Aspartate family	dihydrodipicolinate reductase
BG10262/fliR_at	fliR	2.5	Cellular processes	Chemotaxis and motility	flagellar protein
BG10542/flhA_at	flhA	2.5	Cellular processes	Chemotaxis and motility	flagella-associated protein
BG11711/folD_at	folD	2.5	Biosynthesis of cofactors, prosthetic groups and carriers	Folic acid	methylenetetrahydrofolate dehydrogenase
BG10362/thrS_at	thrS	2.5	Protein synthesis	tRNA aminoacylation	threonyl-tRNA synthetase
BG11792/ileS_at	ileS	2.5	Protein synthesis	tRNA aminoacylation	isoleucyl-tRNA synthetase
BG11834/csbB_at	csbB	2.5	Cell envelope	Biosynthesis and degradation of surface polysaccharides and lipopolysaccharides	stress response protein
BG12644/pfk_at	pfk	2.5	Energy metabolism	Glycolysis/gluconeogenesis	6-phosphofructokinase
BG11533/mcpC_at	mcpC	2.5	Cellular processes	Chemotaxis and motility	methyl-accepting chemotaxis protein
BG10261/fliQ_at	fliQ	2.5	Cellular processes	Chemotaxis and motility	flagellar protein
BG10259/fliZ_at	fliZ	2.5	Cellular processes	Chemotaxis and motility	flagellar protein
BG13939/ytzE_at	ytzE	2.4	Regulatory functions	DNA interactions	similar to transcriptional regulator (DeoR family)

BG10198/ptsG_at	ptsG	2.4	Signal transduction	PTS	phosphotransferase system (PTS) glucose-specific enzyme IIABC component
BG10198/ptsG_at	ptsG	2.4	Transport and binding proteins	Carbohydrates, organic alcohols and acids	phosphotransferase system (PTS) glucose-specific enzyme IIABC component
BG10973/murC_at	murC	2.4	Cell envelope	Biosynthesis and degradation of murein sacculus and peptidoglycan	UDP-N-acetyl muramate-alanine ligase
BG10321/valS_2_at	valS	2.4	Protein synthesis	tRNA aminoacylation	valyl-tRNA synthetase
BG13951/yubB_at	yubB	2.4	Cellular processes	Toxin production and resistance	similar to bacitracin resistance protein (undecaprenol kinase)
BG12840/yerN_at	yerN	2.4	Protein synthesis	tRNA aminoacylation	similar to pet112-like protein
BG13202/yjlC_at	yjlC	2.4	not found	not found	not found
BG10242/fliH_at	fliH	2.4	Cellular processes	Chemotaxis and motility	flagellar assembly protein
BG12012/ycgN_at	ycgN	2.4	Energy metabolism	Amino acids and amines	similar to 1-pyrroline-5-carboxylate dehydrogenase
BG11206/birA_at	birA	2.4	Regulatory functions	Other	transcriptional regulator and biotin acetyl-CoA-carboxylase synthetase
BG13301/ykuQ_at	ykuQ	2.4	Amino acid biosynthesis	Other	similar to tetrahydrodipicolinate succinylase
BG10775/oppF_at	oppF	2.4	Transport and binding proteins	Other	oligopeptide ABC transporter (ATP-binding protein)
BG10273/odhB_at	odhB	2.4	Energy metabolism	TCA cycle	2-oxoglutarate dehydrogenase complex (dihydrolipoamide transsuccinylase, E2 subunit)
BG10076/yaaE_at	yaaE	2.4	Protein synthesis	tRNA aminoacylation	similar to hypothetical proteins
BG11818/yktC_at	yktC	2.4	not found	not found	not found
BG10260/fliP_at	fliP	2.3	Cellular processes	Chemotaxis and motility	flagellar protein
BG10827/gap_at	gap	2.3	Energy metabolism	Glycolysis/gluconeogenesis	glyceraldehyde-3-phosphate dehydrogenase
BG10373/ytxJ_at	ytxJ	2.3	Transcription	Transcription factors	alternate gene name: csb40 similar to general
BG19023/tig_at	tig	2.3	Protein fate	Protein and peptide secretion and trafficking; Protein folding and stabilization	trigger factor (prolyl isomerase)
BG13980/yunF_at	yunF	2.3	not found	not found	not found
BG11873/yvfE_at	yvfE	2.3	Cellular processes	Adaptations to atypical conditions	similar to spore coat polysaccharide biosynthesis
BG13897/ytoI_at	ytoI	2.3	not found	not found	not found
BG10663/grpE_at	grpE	2.3	Protein fate	Protein folding and stabilization	heat-shock protein
BG11501/ypmA_at	ypmA	2.3	not found	not found	not found
BG10700/purE_at	purE	2.3	Purines, pyrimidines, nucleosides and nucleotides	Purine ribonucleotide biosynthesis	phosphoribosylaminoimidazole carboxylase I
BG10820/atpG_at	atpG	2.3	Energy metabolism	ATP-proton motive force interconversion	ATP synthase (subunit gamma)
BG10326/mreB_at	mreB	2.3	Cell envelope	Biosynthesis and degradation of murein sacculus and peptidoglycan	cell-shape determining protein

BG10714/pyrC_at	pyrC	2.3	Purines, pyrimidines, nucleosides and nucleotides	Pyrimidine ribonucleotide biosynthesis	dihydroorotase
BG13965/yueJ_at	yueJ	2.3	Biosynthesis of cofactors, prosthetic groups and carriers	Other	similar to pyrazinamidase/nicotinamidase
BG11607/ypdP_at	ypdP	2.3	not found	not found	not found
BG10201/ptsI_at	ptsI	2.3	Transport and binding proteins	Carbohydrates, organic alcohols and acids	phosphotransferase system (PTS) enzyme I
BG11330/pnp_at	pnp	2.3	Purines, pyrimidines, nucleosides and nucleotides	Salvage of nucleosides and nucleotides	purine nucleoside phosphorylase
BG11701/yqhM_at	yqhM	2.2	not found	not found	not found
BG13117/yitL_at	yitL	2.2	not found	not found	not found
BG10075/yaaD_at	yaaD	2.2	Cellular processes	Detoxification	similar to hypothetical proteins
BG11079/xpt_at	xpt	2.2	Purines, pyrimidines, nucleosides and nucleotides	Salvage of nucleosides and nucleotides	xanthine phosphoribosyltransferase
BG11515/dinG_1_at	dinG	2.2	DNA metabolism	DNA replication, recombination and repair	ATP-dependent helicase
BG13854/ytgD_at	ytgD	2.2	not found	not found	not found
BG10101/metS_at	metS	2.2	Protein synthesis	tRNA aminoacylation	methionyl-tRNA synthetase
BG13433/ymfM_at	ymfM	2.2	not found	not found	not found
BG10280/gerCB_at	gerCB	2.2	Amino acid biosynthesis	Serine family	methyltransferase
BG10280/gerCB_at	gerCB	2.2	Biosynthesis of cofactors, prosthetic groups and carriers	Heme, porphyrin and cobalamin	methyltransferase
BG10280/gerCB_at	gerCB	2.2	Central intermediary metabolism	Other	methyltransferase
BG10280/gerCB_at	gerCB	2.2	DNA metabolism	Restriction/modification; DNA replication, recombination and repair	methyltransferase
BG10280/gerCB_at	gerCB	2.2	Protein synthesis	tRNA and rRNA base modification	methyltransferase
BG12202/ydiE_at	ydiE	2.2	Protein fate	Degradation of proteins, peptides and glycopeptides	similar to glycoprotein endopeptidase
BG12461/ywhG_at	ywhG	2.2	Central intermediary metabolism	Polyamine biosynthesis	similar to agmatinase
BG12771/yceG_at	yceG	2.2	not found	not found	not found
BG10055/soj_at	soj	2.1	Cellular processes	Other	centromere-like function with Spo0J involved in forespore
BG13950/yubA_at	yubA	2.1	not found	not found	not found
BG13046/yheN_at	yheN	2.1	Energy metabolism	Other	similar to endo-1,4-beta-xylanase
BG10783/asd_at	asd	2.1	Amino acid biosynthesis	Aspartate family	aspartate-semialdehyde dehydrogenase
BG12071/ydbD_at	ydbD	2.1	Protein fate	Protein and peptide secretion and trafficking	similar to manganese-containing catalase

BG13901/ytpB_at	ytpB	2.1	not found	not found	not found
BG10100/abrB_at	abrB	2.1	Energy metabolism	ATP-proton motive force interconversion	transcriptional regulator
BG10100/abrB_at	abrB	2.1	Regulatory functions	Other	transcriptional regulator
BG13852/ytgB_at	ytgB	2.1	Transport and binding proteins	Amino acids, peptides and amines; Unknown substrate	similar to ABC transporter (ATP-binding protein)
BG11489/ylxX_at	ylxX	2.1	not found	not found	not found
BG10821/atpD_at	atpD	2.1	Energy metabolism	ATP-proton motive force interconversion	ATP synthase (subunit beta)
BG12473/ywmB_at	ywmB	2.1	not found	not found	not found
BG13476/yoaE_at	yoaE	2.1	Energy metabolism	ATP-proton motive force interconversion	similar to formate dehydrogenase
BG10279/gerCA_at	gerCA	2.1	Biosynthesis of cofactors, prosthetic groups and carriers	Other	heptaprenyl diphosphate synthase component I
BG12796/ydjE_at	ydjE	2.1	not found	not found	not found
BG12864/yetH_at	yetH	2.0	Unknown function	General	similar to hypothetical proteins
BG10392/degS_at	degS	2.0	Regulatory functions	Other	two-component sensor histidine kinase
BG10258/cheY_at	cheY	2.0	Regulatory functions	DNA interactions	two-component response regulator
BG10246/ylxF_at	ylxF	2.0	Cell envelope	Biosynthesis and degradation of surface polysaccharides and lipopolysaccharides	unknown
BG13232/ykfB_at	ykfB	2.0	Energy metabolism	Other	similar to chloromuconate cycloisomerase
BG14065/yvaJ_at	yvaJ	2.0	Transcription	RNA processing; Degradation of RNA	similar to hypothetical proteins
BG11016/penP_at	penP	2.0	Cellular processes	Toxin production and resistance	beta-lactamase precursor
BG10414/murZ_at	murZ	2.0	Cell envelope	Biosynthesis and degradation of murein sacculus and peptidoglycan	UDP-N-acetylglucosamine 1-carboxyvinyltransferase
BG11210/ypjD_at	ypjD	2.0	Transcription	Transcription factors	alternate gene name: jojD
BG14021/yusI_at	yusI	2.0	Energy metabolism	Aerobic; Electron transport	similar to arsenate reductase
BG12597/hisA_at	hisA	2.0	Amino acid biosynthesis	Histidine family	phosphoribosylformimino-5-aminoimidazole carboxamide ribotide isomerase
BG11817/yktB_at	yktB	2.0	not found	not found	not found
BG12772/yceH_at	yceH	2.0	Cellular processes	Detoxification	similar to toxic anion resistance protein

not found, Not found in the JCVI Primary annotation database

Appendix I. Calculation sheet for qPCR.

Rep 1		Rep 2		Rep 3				
Gene	C(t)	Gene	C(t)	Gene	C(t)		**Average**	sd
mbl A	20.37	mbl A	22.46	mbl A	18.58	mbl A	**20.2**	1.6
mbl A	20.33	mbl A	21.17	mbl A	18.26	mbl B	**19.8**	1.8
mbl B	22.08	mbl B	18.94	mbl B	18.15	mbl C	**19.1**	1.1
mbl B	22.21	mbl B	19.32	mbl B	18.33			
mbl C	20.27	mbl C	18.2	mbl C	18.47			
mbl C	20.62	mbl C	18.32	mbl C	18.74			
mbl	N/A	mbl	21.69	mbl	N/A			

Gene	C(t)	Gene	C(t)	Gene	C(t)		**Average**	sd
clpC A	20.54	clpC A	19.92	clpC A	19.13	clpC A	**19.8**	0.7
clpC A	20.52	clpC A	19.46	clpC A	19.06	clpC B	**16.8**	2.6
clpC B	20.14	clpC B	15.8	clpC B	14.74	clpC C	**15.1**	1.6
clpC B	20.05	clpC B	15.72	clpC B	14.43			
clpC C	15.99	clpC C	14.49	clpC C	13.66			
clpC C	17.79	clpC C	14.49	clpC C	13.89			
clpC	N/A	clpC	18.62	clpC	N/A			

Gene	C(t)	Gene	C(t)	Gene	C(t)		**Average**	sd
spoVG A	20.06	spoVG A	20.7	spoVG A	17.29	spoVG A	**19.2**	1.7
spoVG A	19.92	spoVG A	20.6	spoVG A	16.8	spoVG B	**20.3**	2.2
spoVG B	22.67	spoVG B	20.07	spoVG B	17.68	spoVG C	**21.0**	1.6
spoVG B	22.9	spoVG B	20.34	spoVG B	18.11			
spoVG C	22.48	spoVG C	21.26	spoVG C	19.03			
spoVG C	22.65	spoVG C	21.36	spoVG C	19.14			
spoVG	32.01	spoVG	N/A	spoVG	26.5			

Gene	C(t)	Gene	C(t)	Gene	C(t)		**Average**	sd
dnaE2 A	19.14	dnaE2 A	15.56	dnaE2 A	13.52	dnaE2 A	**17.0**	2.3
dnaE2 A	19.55	dnaE2 A	18.18	dnaE2 A	16.33	dnaE2 B	**15.8**	2.4
dnaE2 B	18.65	dnaE2 B	15.12	dnaE2 B	13.17	dnaE2 C	**13.5**	1.8
dnaE2 B	18.77	dnaE2 B	15.58	dnaE2 B	13.48			
dnaE2 C	15.6	dnaE2 C	14.28	dnaE2 C	12.78			
dnaE2 C	15.05	dnaE2 C	11.95	dnaE2 C	11.26			
dnaE2	28.24	dnaE2	29.42	dnaE2	25.53			

mbl standard				**mbl standard**				**mbl standard**			
2 µg/mL		**16 µg/mL**		**2 µg/mL**		**16 µg/mL**		**2 µg/mL**		**16 µg/mL**	
dCt	**fold**	**dCt**	**fold**	**dCt**	**fold**	**dCt**	**fold**	**dCt**	**fold**	**dCt**	**fold**
1.7	3.3	-0.1	0.9	-3.5	0.1	-0.4	0.8	-0.4	0.7	-0.1	0.9
1.8	3.6	0.3	1.2	-3.1	0.1	-4.1	0.1	-0.3	0.8	0.2	1.1
1.8	3.4	-0.1	1.0	-2.2	0.2	-3.0	0.1	-0.1	0.9	0.2	1.2
1.9	3.7	0.3	1.2	-1.9	0.3	-2.9	0.1	0.1	1.0	0.5	1.4
	Av							**Av**			
1.8	**dCt**	**0.1**		**-2.7**	**-2.6**		**-0.2**	**dCt**	**0.2**		
	Av.				**Av.**				**Av.**		
3.5	**Fold**	**1.1**		**0.2**	**Fold**	**0.2**		**0.9**	**Fold**	**1.1**	